TITLE:

ROLE OF CYTOKINES IN THE PATHOGENESIS OF TYPE 1 DIABETES

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TO MY FAMILY

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ABBREVIATIONS

ADCC = Antibody Dependent Cell-mediated Cytotoxicity

ADH = Alcohol Dehydrogenase

AIH = Autoimmune Hepatitis

AMA = Anti-mitochondrial antibody

ANA = Anti-nuclear antibody

A1ATD = α-1-antitrypsin Deficiency

APC = Antigen Presenting Cell

ARA = Anti-reticulin Antibody

ASC = Autoimmune Sclerosing Cholangitis

ASGPR = Asialoglycoprotein Receptor

ASP = Aspartic Acid

AST = Aspartate Aminotransferase

ATCC = American Type Culture Collection

BAF = B Cell Activating Factor

BB = Bio Breeding

BHSE = Biotin Hydroxy Succinimide Ester

BSA = Bovine Serum Albumin

C = Concordant

CAH = Chronic Active Hepatitis

CBU = Comparative Biology Unit

CF-ICA = Complement Fixing Islet Cell Antibodies

CFD = Complement Fixation Diluent

CLD = Chronic Liver Disease

CLDF = Children's Liver Disease Foundation

Con A = Concanavalin A

CTLL = Cytotoxic T Cell Lymphocytes

CV = Coefficients of Variation

D = Discordant

DAB = Diaminobenzidine Tetrahydrochloride

DMSO = Dimethyl Sulfoxide

DNA = Deoxyribonucleic acid

DZ = Dizygotic

EDTA = Ethylenediaminetetra-acetic Acid

ELISA = Enzyme Linked Immuno-sorbent Assay

FCS = Foetal Calf Serum

FITC = Fluorescein Isothiocyanate

GABA = Glutamic Amino Butyric Acid

GAD = Glutamic Acid Decarboxylase

GD = Graves' Disease

GM-CSF = Granulocyte Colony Stimulating Factor

GPC = Gastric Parietal Cell

HBSS = Hank's Balanced Salt Solution

HBV = Hepatitis B Virus

HCV = Hepatitis C Virus

HLA = Human Leukocyte Antigen

IAA = Insulin Autoantibody

ICA = Islet Cell Antibody

ICSA = Islet Cell Surface Antibody

IFL = Immunofluorescence

IFN-γ = Interferon Gamma

IL = Interleukin

IL-1ra = Interleukin-1 Receptor Antagonist

LAF = Lymphocyte Activating Factor

LGL = Large Granular Lymphocytes

LKM = Liver Kidney Microsomal

LMA = Liver Membrane Antibody

LPS = Lipopolysaccharide

LS = Long-standing

LSP = Liver Specific Protein

MAb = Monoclonal Antibody

MHC = Major Histocompatibility Complex

MNC = Mononuclear Cell

MHC = Major Histocompatibility Complex

ml = Millilitre

µl = Microliter

MZ = Monozygotic

NDDG = National Diabetes Data Group

NC = Normal Controls

NIBSC = National Institute for Biological Standards and Controls

NK = Natural Killer

NOD = Non-obese Diabetic

OCT = OCT Compound

OD = Optical Density

OPD = O-phenylenediamine dihydrochloride

PBC = Primary Biliary Cirrhosis

PBMC = Peripheral Blood Mononuclear Cells

PBS = Phosphate Buffered Saline

PEG = Polyethylene Glycol

PHA = Phytohaemoagglutinin

PMA = Phorbol Myristic Acetate

PP = Pancreatic Polypeptide

PSC = Primary Sclerosing Cholongitis

PWM = Pokeweed Mitogen

RD = Recently Diagnosed

RFLP = Restriction Fragment Length Polymorphism

SD = Standard Deviation

SEM = Standard Error of the Mean

SLA = Soluble Liver Antigen

SLE = Systemic Lupus Erythematosus

SMA = Smooth Muscle Antibody

TCGF = T Cell Growth Factor

T_H = T Helper

TNF-α = Tumour Necrosis Factor-alpha

T_c = T Cytotoxic

T_s = T Suppressor

Type 1 diabetes = Insulin Dependent Diabetes Mellitus

Type 2 diabetes = Non-insulin Dependent Diabetes Mellitus

WD = Wilson's Disease

WHO = World Health Organisation

PUBLICATIONS

The following papers have been published or submitted for publication reporting studies contained in the present thesis:

1- HUSSAIN MJ, MUSTAFA A, GALLATI H, MOWAT AP, MIELI-VERGANI G, VERGANI D.
Cellular expression of TNF and IFN-γ in the liver biopsies of children with chronic liver disease. **J Hepatol** (in press).

2- HUSSAIN MJ, PEAKMAN M, LO SSS, VIBERTI GC, WATKINS PJ, LESLIE RDG, VERGANI D.
Elevated serum levels of tumour necrosis factor-α and interleukin-1α presage and accompany the onset of Type 1 (insulin-dependent) diabetes.

3- HUSSAIN MJ, MOWAT AP, MIELI-VERGANI G, VERGANI D.
Serum levels of IL-1, sIL-2R, TNF-α and IFN-γ in children with chronic liver disease.

4- HUSSAIN MJ, PEAKMAN M, WARNOCK T, VERGANI D. Circulating levels of TNF-α and sIL-2R are high in families of patients with IDD.

The following papers have been published or submitted for publication reporting studies unrelated to this thesis, but carried out during the period of Ph.D. studentship of the candidate (1/1990-9/1994):

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Effect of initiation of insulin therapy on T-lymphocyte activation in type 1 diabetes. **Diabetic Medicine** *7*:327.

2- SMERDON RA, PEAKMAN M, **HUSSAIN MJ**, ALVIGGI L, WATKINS P, LESLIE RDG, VERGANI D. (1993)
Increase in simultaneous co-expression of naive and memory lymphocyte markers at diagnosis of insulin dependent diabetes mellitus. **Diabetes** *42*:127.

3- GREALLY P, **HUSSAIN MJ**, SAMPSON AP, PIPER PJ, PRICE JF. (1993)
Sputum TNF-α and leukotrine concentration in cystic fibrosis. **Arch Dis Child** *68*:389.

4- GREALLY P, **HUSSAIN MJ,** VERGANI D, PRICE JF. (1993)
Serum IL-1α and soluble IL-2 receptor concentrations in atopic dermatitis. **Arch Dis Child** *68*:785.

5- SMERDON RA, PEAKMAN M, **HUSSAIN MJ**, VERGANI D. (1993)
Lymphocyte vaccination prevents spontaneous diabetes in the non-obese diabetic mouse. **Immunology** *80*:498.

6- SENALDI G, PEAKMAN M, NATOLI C, **HUSSAIN MJ**, GALLATI H, McMANUS T, VERGANI D, IACOBELLI S.
Relationship between the tumour-associated antigen 90K and cytokines in the circulation of persons infected with human immunodeficiency virus. **J Infection** *28*:31.

7- PETTINGALE KW, **HUSSAIN M,** INAYAT Q, TEE DEH. (1994)
Changes in Immune Status following Conjugal Bereavement. **Stress Medicine** *10*:145.

8- **HUSSAIN MJ**, LAU JYN, WILLIAMS R, VERGANI D.
Hepatic expression of tumour necrosis factor-α in chronic hepatitis B virus infection. **J Clin Pathol** (in press)

ABSTRACT

T lymphocytes and macrophages appear to play an important role in mediating ß-cell damage and causing Type 1 diabetes. Both activated T cells and macrophages operate and interact through the release of soluble factors called cytokines, which influence the type and magnitude of immune responses. It has been suggested that cytokines such as TNF-α and IL-1α can damage the β-cell directly. In Type 1 diabetes, cytokines are likely to have a critical role in individuals whose immune system is unbalanced allowing the emergence of self-destructive processes. To investigate this possibility, sensitive assays to detect a range of cytokines of potential relevance to the immune pathogenesis of diabetes were establised. Using these, serum levels of IL-1α, IL-1β, TNF-α and IL-6 (macrophage-derived cytokines), IFN-γ and IL-2 (T helper 1 cytokine profile) and IL-4 and IL-10 (T helper 2 profile) have been measured in patients with Type 1 diabetes of different disease duration. Increased levels of TNF-α, IL-1α, IL-2 and IFN-γ were found in recently diagnosed patients with Type 1 diabetes when compared with both disease and metabolic control subjects and with normal controls. The presence of this profile of cytokines implies activation of the T_H1 subset of helper cells near to diagnosis of Type 1 diabetes.

After having defined on singleton diabetics which pattern of cytokine alterations occur in Type 1 diabetes, studies were performed in a cohort of non-diabetic identical twins of patients with Type 1 diabetes, some of whom went on to develop the disease themselves, in an attempt to determine whether cytokines could be involved in the pathogenesis of the disease. Increased levels of TNF-α and IL-1α, important mediators of cellular immunity, were detected up to 5 years before the clinical onset of Type 1 diabetes, while cytokine profiles classified as T_H1 (elevated levels of IL-2 or IFN-γ) or T_H2 (elevated levels of IL-4 and IL-10) existed in both prediabetic or non-diabetic twins without dominance of either T_H1 or T_H2. To examine alteration of cytokine levels and their relationship with autoantibodies and HLA alleles, cytokines were investigated in diabetic children and their first degree relatives. Circulating levels of TNF-α and sIL-2R were not only increased in diabetic children but also in their first degree relatives and that increase was not related to autoantibodies or to any specific HLA class II allele (HLA-DR3/4). The study of cytokine secretion by peripheral blood mononuclear cells showed that LPS-stimulated IL-1α and TNF-α secretion by PBMC in vitro are also significantly increased in diabetic children and their siblings and parents when compared with normal controls and that levels of T-cell cytokines, IL-2 and IFN-γ, released after

PHA stimulation, are similar in diabetic children, their siblings and parents, and in normal controls.

Serum levels of cytokines and their cellular expression in the islets of Langerhans of non-obese diabetic (NOD) mice, an animal model of Type 1 diabetes were also studied to define the pattern of cytokine profile involved in this animal model. Serum levels of TNF-α and IL-2 were elevated in diabetic NOD mice when compared with non-diabetic NOD mice and control mice. Both IFN-γ and TNF-α producing cells were present in the islets of Langerhans of diabetic NOD mice.

The involvement of cytokines in the development autoimmune liver disease, an organ-specific autoimmune disease, was investigated in serum as well as in the tissue of the target organ. Serum levels of the cytokines IL-1α, IL-6 and TNF-α were significantly elevated in patients with various forms of chronic liver disease, including those with autoimmune hepatitis, when compared with normal controls. Levels of IFN-γ and sIL-2R were significantly increased in patients with autoimmune hepatitis when compared with other forms of liver disease and normal controls. In contrast, levels of IL-4 were not different between patients with various forms of chronic liver disease and normal controls. Levels of IL-1α, IL-6, TNF-α and sIL-2R were significantly higher in patients with cirrhosis than in patients without. A similar picture was found for disease activity and showed that levels of IL-1α, IL-6, TNF-α and sIL-2R are significantly higher in patients with active disease than those with inactive disease.

Cells producing TNF-α and IFN-γ were present in the liver inflammatory infiltrate of children with autoimmune chronic liver diseases but were rarely seen in metabolic diseases such as Wilson's disease and alpha 1 antitrypsin deficiency.

CHAPTER 1

GENERAL INTRODUCTION

1.1 HISTORY OF TYPE 1 DIABETES

Diabetes mellitus is an ancient disease. The earliest description of its symptoms was found in the Ebers papyrus of Egypt, dating back to 1500 B.C (reviewed by Notkins, 1979 and Schadewaldt, 1989). In the second century A.D. Aretaeus of Cappadocia named the disease diabetes, the Greek word meaning "to flow through a siphon". "Diabetes", he wrote, "is a strange disease that consists of the flesh and bones running together into the urine". In the sixth century, Indian physicians recognised that the urine from diabetic patients was sweet. It was not known until the 18th century, however, that the sweet-tasting substance was identified as the sugar glucose and the word mellitus, or "honeyed" was added.

One of the first clues to the pathology underlying diabetes, came in 1889 when Oscar Minkowski and Baron Joseph von Mering, working in Strasbourg, sought to determine whether the pancreas was essential to life. By careful surgical procedures they removed the pancreas from dogs; the day after operation the caretaker in the laboratory noticed that the dogs' urine attracted an unusual number of flies. In any event Minkowski and von Mering analyzed the urine and found in it high levels of glucose, indicating that the removal of the pancreas gave rise to a syndrome resembling diabetes. This finding strongly implied that the pancreas was secreting a substance that regulated the metabolism of glucose. All attempts (Minkowski in 1890, Goldscheider in 1894, Cohnheim in 1903 and others) to alleviate experimental diabetes by feeding pancreatectomized dogs raw pancreas or by injecting them with crude pancreatic extract, however, were unsuccessful (reviewed by Schadewaldt, 1989).

The existence of insulin was not known until two Canadian investigators, Frederick G. Banting and Charles H. Best, extracted insulin from dog pancreas. The first patient to receive the active extract prepared by Banting and Best was Leonard Thompson aged 14 who appeared at the Toronto General Hospital with blood glucose levels of 500 mg (Banting et al., 1922). Extracts were first administered on January 11, 1922. These authors induced a reduction in the concentration and excretion of blood glucose. News of the experiment spread rapidly, and in a short time insulin was being widely and successfully employed to treat the acute symptoms of diabetes mellitus in patients. Until recently, virtually all insulin for therapeutic use was produced from porcine and bovine pancreas. The antigenicity of animal insulin led several clinicians to believe that human insulin might be of more benefit to diabetic patients (Yalow and Berson, 1961). Human insulin was introduced later and was originally prepared by extraction from cadaveric pancreas

(Nicol and Smith, 1960). Human insulin is now manufactured on a large scale by two fundamentally different processes involving enzymatic conversion of porcine insulin (Owens *et al*., 1986; Pickup, 1986). Alternatively human insulin is biosynthesized using recombinant DNA technology (Brange *et al*., 1988 and 1990). Although the treatment with insulin allows patients with diabetes to live an almost normal life, it is not able to reduce the prevalence of long-term diabetic complications. It may be that in the future, after knowing the cause of the disease, alternative approaches, such as immunotherapy and islet cell transplantation, will replace traditional insulin treatment.

TERMINOLOGY

Diabetes is not a single disease but rather a heterogeneous group of diseases that lead to an elevation of glucose in the blood. Chronic hyperglycaemia and the risk of developing complications are the two unifying properties which have held the notion of diabetes together. During the past decades, however, there has been remarkable progress in understanding diabetes. In 1951, RD Lawrence described "two types of diabetes mellitus, with and without plasma insulin" (Bornstein and Lawrence, 1951). The two main nosological entities recognised under the name of diabetes have been labelled in turn "diabète maigre" and "diabète gras", "juvenile onset type" and "maturity onset type" and more recently "insulin dependent" and "non-insulin dependent diabetes mellitus" (Type 1 and Type 2 diabetes) (reviewed by Keen and Tang Fui, 1982; Keen, 1986). The purely descriptive terms of Type 1 diabetes and Type 2 diabetes have now been equated by the National Diabetes Data Group (NDDG) and the Expert Committee on Diabetes of the World Health organisation (WHO) with the terms "type 1" and "type 2" to denote their distinct pathogeneses (National Diabetes Data Group 1979; WHO Technical Report series 1980 and 1985).

1.2 DIVERSITY OF DIABETES

In his Claude Bernard lecture delivered to an audience of European diabetologists in 1979, DA Pyke noted that there was no longer serious need to debate the proposition that insulin dependent and non-insulin dependent diabetes are two distinct entities (Pyke, 1979). The evidence then available consisted of the following compelling arguments:

(1) Type 1 diabetes and Type 2 diabetes are different histologically. A striking feature of Type 2 diabetes is the relative normality of the islets compared to Type 1

diabetes where they are heavily infiltrated by mononuclear white cells, in a process known as insulitis (Gept, 1965).

(2) If Type 2 diabetes were due to a degenerative process in the β-cell it would presumably progress, eventually leading to insulin dependence; yet this is a rarity. More often these patients can be controlled without insulin. This static situation in Type 2 diabetes is in striking contrast to the insulin dependent type where a destructive process affecting the islets leads to complete failure of insulin production.

(3) Islet cell antibodies are commonly found in newly diagnosed cases of Type 1 diabetes, rarely in Type 2 diabetes.

(4) Type 1 diabetes is associated with certain HLA alleles, whereas Type 2 diabetes is not.

(5) Identical twin studies show widely different concordance rates in the two types.

The different and often conflicting findings about the nature of diabetes and its heterogeneity have provoked the establishment of strict criteria for its diagnosis and classification. The NDDG classification of diabetes separates insulin dependent (type 1) patients from those who are non-insulin dependent (type 2) irrespective of age. A third group of patients fall in the "other types" category, which includes hyper-glycaemia associated with other forms of disease such as pancreatic diseases, hormonal abnormalities, drug or chemical induced diseases and a variety of genetic syndromes.

Type 1 diabetes (insulin dependent) is characterised by severe and pathognomonic changes in the pancreatic islets, by an eventual absolute deficiency of endogenous pancreatic insulin secretion, insulinopaenia, proneness to ketosis and a dependency on daily insulin administration for the maintenance of life. It can be developed at any age and its onset is more abrupt than type 2 (reviewed by Skyler and Cahill, 1981).

Type 1 diabetes appears to be heterogeneous in terms of genetics and environmental factors that precipitate the disease (Rotter and Rimoin, 1978). Genetic determinants are thought to be important in most patients, as expressed by the associated increased frequency of certain histocompatibility antigens on chromosome 6 (Nerup, 1976; Cudworth and Woodrow, 1976). Abnormal immune responses and autoimmunity are also thought to play an aetiologic role, and islet cell antibodies are frequently present at diagnosis in this type of diabetes (reviewed by Irvine, 1979).

Type 2 (non-insulin dependent) diabetes, frequently presents with minimal

or no symptoms referrable to the metabolic aberrations of diabetes and is the much more prevalent type. It is characterised by retention of endogenous pancreatic insulin secretion, although with altered secretory dynamics; the absence of ketosis; and insulin resistance due to diminished target cell response to insulin. Patients with non-insulin dependent diabetes mellitus are usually not dependent on insulin for prevention of ketosis or maintenance of life, although they may require insulin for correction of symptomatic or persistent fasting hyperglycaemia, if this cannot be achieved with the use of diet or oral agents (reviewed Skyler and Cahill, 1981). The whole range of insulin responses to glucose, from low to supranormal has been reported to be associated with insulin resistance (Reaven, 1984). Type 2 diabetes also has a genetic basis, which appears to be stronger than in type 1 diabetes, as evidenced by a more frequent familial pattern of occurrence (Fajans *et al*., 1978). Environmental factors superimposed on genetic susceptibility are undoubtedly involved in the onset of the disease. Some of the patients with this type of disease may develop insulin dependent diabetes later. Characteristic aggregation of HLA types and islet cell antibodies have not been found in patients with type 2 diabetes.

1.3 PATHOGENESIS OF TYPE 1 DIABETES

By the time Type 1 diabetes appears, most of the β-cells in the pancreas have been destroyed. Gepts (1965) observed in his seminal study different lesions in patients with type 1 diabetes. He concluded that these represent different stages of the same destructive process. It was suggested later that the destructive process is relatively slow and it might take years for diabetes to develop (Tarn et al., 1987).

1.3.1 PATHOLOGICAL CHANGES

A lymphocytic infiltration in and about the islets of Langherans was first noted in 1910 by Opie and Weichselbaum. They reported that: "These cells are small lymphocytes with an occasional polymorphonuclear cell but without eosinophils or plasma cells". This lesion was later to be named as "insulitis" by Von Mayenburg in 1940. Subsequently, in 1952, Ware and Le Compte reported that: "This type of change is particularly apt to be found in a young individual and is associated with severe diabetes". Later, Gepts provided a major contribution in the understanding of the pathology of Type 1 diabetes, by observing that islet inflammation is never a uniform lesion (Gepts, 1965).

Gepts (1965), in his study, observed three types of lesions. The first type is insulitis, which is presently considered the hallmark of Type 1 diabetes, but it is not

the most frequent lesion observed at the clinical onset of the disease. The second type is the pseudoatrophic islet, the most common lesion, which is small, inconspicuous and completely devoid of insulin producing β-cells; however, it still contains cells secreting glucagon, somatostatin and pancreatic polypeptide. No inflammatory cells are present in pseudoatrophic islets. The heavy mononuclear cell infiltrate is present in fewer and better preserved islets, where the β-cells are mainly surrounded by lymphocytes. The third type is an intermediate lesion between insulitis and pseudoatrophic islets which is also described where islets contain decreasing amount of β-cells and fewer numbers of inflammatory mononuclear cells. Disappearance of β-cells is accompanied by disappearance of inflammatory cells. Appearance of foci of β-cell regeneration is associated with reappearance of lymphocytes in large numbers.

1.3.2 GENETIC SUSCEPTIBILITY

It has been known for some time that diabetic patients frequently have a family history of the disease i.e. the disease is present among relatives of diabetics. The familial aggregation is more common in Type 2 diabetes than in Type 1 diabetes (Gamble, 1980; Rotter and Rimoin, 1981).

The evidence that Type 1 diabetes is not entirely genetic in its origin came from the world's largest twin study by Pyke and collaborators (Pyke, 1979). Initially, this study reported a concordance rate for Type 1 diabetes of 50% (Tattersall and Pyke 1972; Pyke, 1979), but a subsequent analysis of an extended series of twins suggests a figure of 36% (Leslie and Pyke, 1986); smaller studies show concordance of 20% (Gottlieb and Root, 1968; Cahill, 1979). These observations suggest that disease susceptibility is inherited and that another factor, presumably environmental, converts this susceptibility into clinical disease.

The association between the HLA region and Type 1 diabetes came from a study on families of affected individuals which showed that type 1 diabetes present in family members segregates with particular HLA haplotypes (Cudworth and Woodrow, 1975). Subsequent studies found a marked excess of affected sib pairs who were HLA identical (i.e., both HLA haplotypes had been inherited in common), implying that genes influencing the predisposition to diabetes are often inherited from both parents (Cudworth, 1978). Other authors confirmed these observations, but considerable argument has taken place concerning the mode of inheritance of the HLA susceptibility factor and as to whether it is dominant or recessive (Barbosa et al., 1977; Bengsch et al., 1978). Thus, Barbosa et al. (1980) selected patients in order

to identify two sets of families: those with horizontal and those with vertical aggregation. These authors assumed that the recessive inheritance operates for the first set while dominant inheritance operates for the second (Barbosa et al., 1978 and 1980). This hypothesis has been criticised by Suarez and Van Eerdewegh (1981) who found these different aggregation patterns did not reflect true differences in mode of inheritance but rather differential selection of families with an aggregation of diabetes susceptibility genes (Suarez and Van Eerdewegh, 1981).

Recent combined analyses of a large number of Type 1 diabetic families with two or more affected siblings concluded that the HLA region on human chromosome six encodes one (or more) susceptibility genes(s) (reviewed by Todd, 1990). These genes contribute 30 to 60% of genetic susceptibility to Type 1 diabetes (Rotter and Landaw, 1984; Risch, 1987).

1.3.3 HLA

The major histocompatibility complex (MHC) was recognized by its major influence on transplantation rejection and by international agreement the human MHC has been labelled HLA (Human Leukocyte Antigen) (Roitt, 1988).

The genes coding for the HLA antigens are on chromosome 6 and form three main classes. Class I molecules consist of one heavy and one light polypeptide chain and are the products of three reduplicated loci: HLA-A, HLA-B and HLA-C. These loci are paired in each individual and each of these chromosome sets is termed a haplotype. Class I molecules are found on almost every nucleated cell in the body and transcribed from both the maternal and paternal chromosomes.

The genetic region coding for MHC class II molecules is called HLA-D region, this includes DP, DQ and DR antigens. Their distribution is more limited than that of class I molecules: they are expressed constitutively only on B lymphocytes, monocytes/ macrophages, dendritic cells, thymic epithelial cells and on activated but not resting T cells (Winchester and Kunkel, 1980).

MHC class III molecules comprise a group of complement components. These are C2 and C4 of the classical pathway and Bf, coding for factor B of the alternative complement pathway. They are proteins found in serum rather than on cell surfaces. Unlike class I and II molecules, MHC class III molecules do not appear to control the immune response nor do they serve as transplantation antigens (Roitt, 1988).

The genetic association of HLA antigens with Type 1 diabetes was initially described in 1974 by Cudworth and Woodrow in Britain and Nerup and colleagues

in Denmark. They found that the class I HLA antigens, B8 and B15 are two or three times more common in Type 1 diabetics than in Type 2 diabetics. The association between Type 1 diabetes and class II antigens/complex (HLA-D/DR locus) was reported later by Barbosa *et al*. (1979) and Sachs *et al*. (1980) but the information was limited. In 1982, Cudworth and Wolf reported that Type 1 diabetes was even more closely associated with the class II HLA antigens D/DR3 and D/DR4, in which the genes for the B8 and B15 are in linkage disequilibrium.

The possibility that more than one gene in the HLA complex determines susceptibility to Type 1 diabetes has been suggested by a Danish group (Svejgaard et al., 1975). Subsequent studies by Bottazzo and Doniach (1976), and Irvine (1977) who proposed that Type 1 diabetes could be subdivided into autoimmune and viral-induced types, with an intermediate group in Irvine's classification. Rotter and Rimoin (1978) proposed further heterogeneity based on differential immunological correlations with different HLA phenotypes. These authors postulated that Type 1 diabetes associated with HLA B8-DR3 is a disease distinct from Type 1 diabetes associated with HLA B15-DR4, the first being an autoimmune form, the second representing an "insulin antibody responder type", characterised by a vigorous humoral immune response to exogenous insulin.

Studies of restriction fragment length polymorphism (RFLP) in the HLA-D (class II) region suggest that variation in the DQ-β region may be more strongly associated with risk for Type 1 diabetes than is variation in the DR region, at least as regards the HLA-DR4 associated susceptibility (Owerbach et al., 1983; Monos et al., 1987; Todd et al., 1987; Nepom, 1990). This variant called the DQ-β w3.2 allele, occurs in as many as 90-95% of those Type 1 diabetes who carry the HLA-DR4 allele and about 60-75% of HLA-DR-carrying of non-diabetic controls (Monos et al., 1987). Todd et al. (1987) in their molecular studies focused on the DQ-β region, found also a difference between haplotypes which are and are not associated with increased risk for Type 1 diabetes. Those haplotypes which are not associated with increased risk all coded for the amino acid residue aspartic acid (ASP) at position 57 of the DQ-β chain, while the "high risk" haplotypes, including DR4/DQ-β w3.2, DR3/DQ-β w2, DR1/DQ-β w1.1 and DR2/DQ-β w1.AZH, were all associated with residues other than ASP at position 57 (Todd et al., 1987). It was then concluded that the disease susceptibility is most closely associated and linked to the HLA-class II region, containing the HLA-DR and DQ loci (reviewed by Todd, 1990).

Protection from Type 1 diabetes may be conferred by genes in the HLA region. These include DR2 (Platz et al., 1981) and a 3.7 kb DQ-β fragment

(Owerbach et al., 1983), the latter being protective when carrying an ASP at position 57 (ASP 57-positive) (Todd *et al*., 1987). The implication of both DQ-α1 and DQ-β1 in HLA-associated protection from Type 1 diabetes has been investigated recently by Cavan et al. (1993). These authors found that the DQ-α1 and DQ-β1 alleles linked with DR2, DRw6 and DR7 haplotypes are associated with protection from diabetes in British Caucasoid, and the presence of aspartate at position 57 is unlikely, on its own, to be an important determinant of disease protection (Cavan et al., 1993). The role of HLA in the aetiology of Type 1 diabetes has been challenged by a number of studies, including that by Pyke noted above demonstrating that concordance rates of Type 1 diabetes in monozygotic twins are 50% or less (Pyke, 1979). This evidence does not support HLA as being the primary familial susceptibility determinant in Type 1 diabetes and suggests that the inherited component is disease susceptibility. Thus, so far as Type 1 diabetes is concerned, HLA genotyping is of limited value in genetic counselling and can be justified only in terms of assessment of disease susceptibility. Attempting to predict the incidence of type 1 diabetes by HLA-typing individuals without studying other factors which are involved in the predisposition of the disease has no practical value.

Studies on identical twins (genetically similar) especially those discordant for diabetes (one twin is diabetic and his co-twin is not) may provide information regarding the relative role of genetic and environmental factors in developing of Type 1 diabetes.

1.3.4 TWIN STUDIES

Twins studies represent one approach to resolving the question whether familial aggregation of a disease can be due either to genetic or environmental factors (Rimoin and Rotter, 1984). The frequency of concordance (both members of the twin pair affected) of monozygotic (identical) twins is compared with that of dizygotic (fraternal) twins. Monozygotic twins share all genes, and thus theoretically should be concordant for disorders with purely genetic aetiology. Dizygotic twins share only half their genes and are thus no more alike genetically than any pair of sibs. The research, conducted by David Pyke and his colleagues at King's College Hospital in London, on diabetic twins was started in early 1970's (Tattersall and Pyke, 1972). More than a hundred pairs of identical twins were examined, the largest series of twins ever studied. They found that when one twin of a pair developed diabetes after the age of 50 years, the other twin developed the disease within several years in almost every case. If one twin developed the disease before the age of 40,

however, the other twin developed it in only half of the cases. They found also that the majority of the twins over 40 had Type 2 diabetes, whereas most of those under 40 had Type 1 diabetes. It was then suggested that there are at least two types of diabetes: one mainly inherited (Type 2 diabetes), the other mainly environmental (Type 1 diabetes).

The evidence accumulated by the King's twin study, was extended and reassessed in 1979 when the number of identical twin pairs recruited into that study was 185 (Pyke, 1979). In most concordant pairs, twins became diabetic at about the same time within 5 years in two thirds (66%) and within 10 years in nine tenths (10%). On the other hand in the discordant pairs, a third (33%) had gone for more than a 10 year interval since the first twin developed diabetes and the second twin was still not diabetic. The conclusion of this study was that in many, and probably most, discordant pairs in which the first twin had Type 1 diabetes for more than 5 years the second twin would always remain unaffected. This highlights non-genetic factors, probably environmental, in the initiation of the disease (Olmos et al., 1988).

1.3.5 ENVIRONMENTAL FACTORS

Viruses and, to a much lesser extent, toxins are the environmental factors that have been recurrently implicated in the development of Type 1 diabetes. Epidemiological studies have confirmed the relevance of environmental factors to the induction of Type 1 diabetes. Observation on the onset of Type 1 diabetes in various seasons of the year forms the first body of circumstantial evidence. The possible role of viruses in the cause of diabetes was suggested before the turn of the century when its association with mumps was first reported. In the early 1920s, Gunderson noted an increased prevalence of diabetes in several Scandinavian villages after outbreaks of a mumps virus infection, and in a subsequent publication, he asked: "Is diabetes of infectious origin?" (Gunderson, 1927). Although this question remains unanswered, intriguing information has accumulated supporting the view that viruses may be aetiologically responsible for at least some cases of the disease (Gamble and Taylor, 1969; MacMillan et al., 1977). Circumstantial evidence connecting viruses with Type 1 diabetes came initially from an association of certain childhood viral infections with the onset of the disease.

The appearance of Type 1 diabetes may coincide with mumps (Kremer, 1947; Hinden et al., 1962; Messaritakis et al., 1971), rubella (Forrest et al., 1971; Smithsells et al., 1978), cytomegalovirus (Hultqist et al., 1973) and Epstein-Barr virus infection (Burgess et al., 1974). In 1968, Gamble et al. reported an association

between infection with Coxsackie virus group B, type 4, and Type 1 diabetes (Gamble et al., 1969). The claim of these investigations was based on the demonstration of an increased prevalence, and high titre, of anti-viral antibodies in diabetic subjects, compared to normal controls, though a subsequent study failed to demonstrate differences in the antiviral antibody titre in identical twins discordant for Type 1 diabetes (Nelson *et al*., 1975). An increased frequency of IgM antibodies to Coxsackie B1-6 was also found in a subsequent study in children, 2-16 weeks after the onset of Type 1 diabetes (King et al., 1983). The presence of Coxsackie B virus IgM (CBV-IgM) was found not only in children with newly-diagnosed Type 1 diabetes, but also in a recent study, in their siblings (Frisk et al., 1992). When a high cut-off value was used, 16/35 (46%) diabetic children and 16/47 (34%) siblings showed positive CBV-IgM antibodies. The first direct evidence that viruses are capable of causing diabetes in human beings came in 1979 by Yoon and his colleagues. These authors isolated a Coxsackie B4 virus from the pancreatic tissue of a child with meningo-encephalitis and diabetes of sudden onset (Yoon et al., 1979). Subsequent studies of the pathological findings in the child's pancreas, however, indicated that extensive β-cell damage with features of chronicity might have preceded the infection (Gepts and Lecomte, 1984). It was then recognised after reviewing studies on the pathogenesis of Type 1 diabetes that the disease develops over years, with viruses representing environmental triggering events, preceding the onset of Type 1 diabetes (Bottazzo et al., 1985).

The mechanism by which a virus might initiate autoimmunity against beta cells is now the subject of considerable speculation. One possible mechanism by which viruses, and in particular the CBV, produce diabetes may be by molecular mimicry (Rewers et al., 1987; Bae et al., 1990). In molecular mimicry, sequence and epitope homologies between viral antigens and host determinants result in the generation of host-specific immune responses (Reviewed by Szopa et al., 1993b). It has been noted that there was an extensive similarity between the GAD65 proteins (glutamic acid decarboxylase, the biosynthetic enzyme of γ-amino-butyric acid [GABA], high levels of which are expressed in beta cell) and the P2C protein of CB4 virus (Kaufman et al., 1992; Bu et al., 1992).

Alternatively, viruses may alter β-cell surface antigens, marking the cell as foreign tissue and targeting it for immunological destruction (Oldstone et al., 1991). Viruses could also induce the expression of HLA class I and II antigens on the surface of β-cells, thereby initiating their destruction, or induce cytokines such as interferon, interleukins and tumour necrosis factor, thereby altering the immune

response by regulating the expression of β-cell antigens and thus contributing to tissue inflammation (Campbell et al., 1988; Cavallo et al., 1994).

Toxins can also kill β-cells and give rise to diabetes. Streptozotocin is of particular interest because it is directly β-cytotoxic and capable of inducing diabetes (Like and Rossini, 1976). Further studies indicated that high doses of streptozotocin is directly toxic to the β-cells but seems to induce an autoimmune type of diabetes when given as multiple small doses to certain strains of mice (Kolb et al., 1987).

A food additive, such as N-nitroso-compounds, a component of processed mutton was also reported to cause ketosis-prone diabetes in Iceland (Helgason and Jonasson, 1981).

Although environmental factors are involved in the pathogenesis of Type 1 diabetes, they seldom act alone. They act with other factors such as genetic elements including the genes within and without the HLA region. An insight on how such diverse elements interact to produce Type 1 diabetes can be gained by studying animal models of the disease.

1.3.6 ANIMAL MODELS OF TYPE 1 DIABETES

Major advances in the field of autoimmunity have been achieved using animal models. These allow the natural history of a disease to be studied, including the subclinical prodrome; they allow manipulation of breeding and rearing to study genetic and environmental influences; and they allow experimentation with therapies.

By the end of 1980 more than 10 strains of spontaneously diabetic rodents were established as useful experimental animals (Makino *et al*., 1980). One of the most well known animal models bearing a striking resemblance to human Type 1 diabetes is the non-obese diabetic (NOD) mouse.

The NOD mouse, also called the Tochino mouse, was derived from a sub-line of the CTS strain in Japan, and has been described by Makino et al. (1980). About 80% of females and 20% of males become diabetic. The animals are lean, ketosis prone, and develop pancreatic insulitis around 4-5 weeks after birth. Following insulitis, total absence of pancreatic β-cells is found, which results in insulinopaenia (Kano et al., 1986). The finding of lymphocytes infiltrating islet β-cells suggested that diabetes in NOD mice is an immune-mediated disease. The autoimmune origin of the disease has been further documented by the fact that purified T cells from diabetic mice can adoptively transfer diabetes into intact newborn, or irradiated adult recipients (Bendelac et al., 1987; Miller et al., 1988).

Affected mice usually bear a unique class II MHC gene (Hattori et al., 1986), and have reduced numbers of T lymphocytes, natural killer cells and cytotoxic lymphocytes (Kataoka et al., 1983). Autoantibodies such as islet cell surface antibodies (ICSA), intra-cytoplasmic antigens (ICA) (Yokono et al., 1984; Hari et al., 1986) and insulin autoantibodies (Michel *et al*., 1989) are also detected in sera of NOD mice. However, autoantibodies against glutamic acid decarboxylase (GAD) in the NOD mouse are absent and it has been demonstrated recently that GAD is expressed at low level in mouse islets (Velloso et al., 1993).

Thus the NOD model may provide a unique opportunity to evaluate the respective contribution of cell-mediated and humoral immune responses in a spontaneous organ-specific autoimmune disease.

1.4 TYPE 1 DIABETES "AN IMMUNE MEDIATED DISEASE".

Eisenbarth (1986) concluded in his seminar given at the Beth Israel Hospital, Boston, after evaluating the information available, that there is sufficient evidence to support the contention that Type 1 diabetes is an immune mediated disease (Eisenbarth, 1986).

A variety of humoral and cellular immune changes have been detected in newly diagnosed patients with Type 1 diabetes, including production of autoantibodies to pancreatic islet cells, increased number of activated T lymphocytes and alterations in number and function of immunoregulatory T lymphocytes and natural killer cells (reviewed by Vergani, 1987a). Thus, evidence exists supporting the concept that Type 1 diabetes is an autoimmune disease.

1.4.1 STUDIES ON CELLULAR IMMUNITY IN TYPE 1 DIABETES

The involvement of lymphocytes in the pathogenesis of Type 1 diabetes has been proposed on the basis of numerous observations. In 1971, Nerup et al. first reported inhibition of migration of leukocytes specific for the endocrine pancreas in Type 1 diabetes (Nerup et al., 1971). Subsequent studies confirmed this observation, using the leucocyte migration inhibition test for *in vitro* detection of cellular (delayed type) hypersensitivity in man (McCuish et al., 1974).

T cell involvement in the pathogenesis of Type 1 diabetes has been documented in several studies. The presence of T cells was first detected by Bottazzo et al (1985) in the pancreatic islets (insulitis) of a child who died soon after the onset of diabetes. This author found that the predominant cells were T cells and the majority of these were CD8 positive. In addition, the disease can be transferred

with purified T cells from diabetic NOD mice to healthy newborn animals (Bendelac et al., 1987). Prevention of the disease by T cell depletion or inactivation has also been achieved by neonatal thymectomy in the BB rat (Like et al., 1982) and the NOD mouse (Tochino, 1986).

The availability of monoclonal antibodies detecting lymphocyte surface markers has attracted many investigators to study the possibility that Type 1 diabetes is associated with an imbalance in the relative proportion of T lymphocyte subsets. Mature T lymphocytes are divided into two main populations, expressing other CD4 and CD8 molecules (Blue et al, 1985). CD4-expressing T lymphocytes are immunoregulatory cells frequently termed helper or helper/inducer in view of their ability to initiate, promote and enhance the functions of other immune cells such as B lymphocytes and macrophages (Reinherz and Schlossman, 1980). CD8 expression is detectable on approximately one third of peripheral blood T cells, and in functional terms these cells have come to denote cells with a suppressor or cytotoxic function (Gatenby *et al*., 1982). CD4 and CD8 molecules bind to class II and I HLA molecules respectively, stabilising the interaction between the T cell receptor and antigen held within the cleft of the HLA molecule on the antigen presenting or target cell (Doyle and Strominger, 1987; Norment et al., 1988). More recently, T lymphocytes have been divided according to their patterns of secretion of cytokines (Mosmann et al., 1986; Romagnani, 1991). This approach was established for CD4+ lymphocytes initially. Interleukin-2 and IFN-γ secretion defines cells involved in delayed-type hypersensitivity reactions (T_H1) while T_H2 cells, involved in activating B lymphocytes, secrete IL-4, IL-5 and IL-10.

Several studies have detected abnormal numbers of the main CD4 and CD8 functional T lymphocytes subsets at diagnosis of Type 1 diabetes, but there is no consistent picture as to the direction of the change. Both increased, decreased and normal levels of CD4+ lymphocytes have been described (Galluzzo et al., 1984; Herold et al., 1984; Scheinin et al., 1988). A slightly clearer picture has been obtained in studies of CD8+ lymphocytes, which appear to be reduced at diagnosis in most (Galluzzo et al., 1984; Scheinin et al., 1988) but not all (Herold et al., 1984) studies. The study of CD8+ T lymphocytes in twin populations with Type 1 diabetes has indicated that the reduction may be genetically determined since it was detected in diabetic twins of pairs concordant for the disease and also in non-diabetic co-twins from long term discordant pairs in whom diabetes is unlikely to develop (Johnston et al., 1988). A reduction in CD8+ lymphocytes would be consistent with one of the classic theories of the pathogenesis of autoimmune disease, namely that a

reduction in the suppressor mechanism may facilitate the appearance of autoimmunity but it is most unlikely to be the direct cause of Type 1 diabetes (Cooke, 1988).

Increased levels of circulating activated (HLA-DR+) T lymphocytes at the diagnosis of Type 1 diabetes have been a consistent finding (Jackson et al., 1982; Pozzilli et al., 1983; Alviggi et al., 1984; Scheinin et al., 1988). They are present before insulin treatment (Peakman et al., 1990) and have a tendency to decline as time from diagnosis increases (Alviggi et al., 1984).

In addition to T cells, macrophages have been demonstrated in insulitis lesions. In the BB rat, the appearance of macrophages in islets of Langerhans is the first event heralding the onset of insulitis (Walker et al., 1988; Hanenberg et al., 1989). Manoeuvres designed to inhibit macrophages function, such as administration of silica, inhibit the development of diabetes in the BB rat (Oschilewski et al., 1985). Macrophages have also been detected in the pancreatic islets in man when pancreas from patients with Type 1 diabetes was available for histological examination at diagnosis (Foulis et al., 1991).

The most convincing evidence of the involvement of the immune system in the pathogenesis of Type 1 diabetes was provided by surgeons and not by immunologists! Sutherland and colleagues (1984) transplanted a portion of pancreas from four identical unaffected twins into their diabetic co-twins. Diabetes was initially cured but a few weeks after transplant the disease recurred. The histological examination of the transplanted pancreas revealed heavy lymphocytic infiltration of the islets and 90% of infiltrating lymphocytes were positive for the CD8 marker, which is present on cytotoxic and suppressor T lymphocytes (Sutherland et al., 1984). Since the twins were genetically identical this cannot be the result of tissue rejection and indeed the exocrine pancreas was not involved in the inflammatory process. These observations indicate that patients with Type 1 diabetes have circulating memory T-lymphocytes which are sensitised to β-cells and capable of destroying them.

This work by Sutherland provides the strongest evidence currently available to answer the question of whether the immune system is connected with the development of Type 1 diabetes.

1.4.2 STUDIES ON HUMORAL IMMUNITY IN TYPE 1 DIABETES

The discovery of islet cell antibodies (ICA) by Bottazzo et al. in 1976 first implicated autoimmunity in Type 1 diabetes (Bottazzo et al., 1976). Further studies

by others such as Lendrum et al. (1976), Del Prete et al. (1977), Lernmark et al. (1987) and Riley et al. (1990) confirmed his observation. These authors found that ICA can be detected in 60-70% of newly diagnosed Type 1 diabetic patients and in 5-8% of first-degree relatives. Immunofluorescence studies show that the antibody reacts with the cytoplasmic components of all four cell types in
the islets (α, β, δ and pp)* indicating the lack of specificity for the β-cell (Dobersen et al., 1980). In 1980, Bottazzo et al. described complement fixing islet cell antibodies (CF-ICA) which seem to be more specific markers of islet-cell damage, probably because some of them react exclusively with β-cells (Bottazzo et al., 1980). At diagnosis of Type 1 diabetes, CF-ICA is present in fewer patients than conventional ICA. Since CF-ICA is directed against cytoplasmic and not membrane antigens, attempts were made to identify antibodies directed against surface membrane of β-cells. Circulating antibodies against the surface of rat islet cells were detected in children with Type 1 diabetes by Lernmark et al. (1978). These authors found that islet cell surface antibodies (ICSA) were present in 32% of children tested after the onset of the disease (1 day-5 years) (Lernmark et al., 1978) and, in a subsequent study, approximately 67% had a positive reaction at diagnosis, but this percentage declined thereafter (Lernmark et al., 1981). ICSA, however, is present only in about half of the patients with Type 1 diabetes and up to 8% of healthy individuals, thus putting its pathogenic role in doubt (Gleichmann and Bottazzo, 1987).

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* The islets of Langerhans are composed of at least four different types of islet cells: the insulin-secreting β cells, the glucagon-secreting α cells, the somatostatin-secreting δ cells, and a fourth type of cells which have been shown to secrete pancreatic polypeptide (pp) and are therefore designated as pp cells. They have a distinct pattern of localization. For example, in the islets of the tail, the body and the ventral part of the head, β cells represent the predominant type of cells (71%), followed by α cells (20%), D cells (8%) and PP cells (<2%).

Another autoantibody found in Type 1 diabetes is the insulin autoantibody (IAA) (Palmer et al., 1983). IAA has been detected in 38% newly diagnosed patients before treatment with insulin and in 47% of unaffected identical twins of Type 1 diabetes (Wilkin et al., 1985). IAAs appear to have little prognostic significance in the absence of ICAs, however, these are present in combination in 50% of those who develop Type 1 diabetes and are highly predictive (Ziegler et al., 1989; Atkinson et al., 1986). It has been suggested that anti-insulin antibodies may originate from insulin receptor antibodies in accordance with the idiotype anti-idiotype mechanism (Cooke et al., 1984). Although IAA is directed at a β-cell specific product, insulin, it is unlikely to be the initiator of β-cell damage, since insulin has a protected location within granules in the β-cell, a site surely inaccessible to the immune system.

In the early 1980, Baekkeskov et al. reported that sera from newly diagnosed diabetic children consistently immunoprecipitated a human pancreatic islet cell protein having a molecular weight of 64 kilodaltons (64 kD) (Baekkeskov et al., 1982). Antibodies to a 64 kD protein are associated with Type 1 diabetes and have been detected years before the onset of symptoms. They are also present in the two animal models of Type 1 diabetes, the NOD mouse and the BB rat (Baekkeskov et al., 1984; Atkinson and Maclaren, 1988; Atkinson et al., 1990). A 64 kD islet cell protein identified as glutamic acid decarboxylase (GAD) (Baekkeskov et al., 1990) responsible for the synthesis of γ-amino-butyric acid (GABA) was found in the brain, peripheral neurons, the pancreas and other organs (Baekkeskov et al., 1990; Erdö and Wolff, 1990). Autoantibodies to a 64 kD protein appear to be more predictive for diabetes than insulin or islet cell cytoplasmic antibodies which can be detected years before symptoms of Type 1 diabetes (Atkinson et al., 1990).

Antibodies to other protein fragments of 50 kD, 40 kD and 37 kD have been reported in patients with new-onset type 1 diabetes (Christie et al., 1990). Antibodies to the 50 kD fragment are found in most Type 1 diabetic patients (81%) and is positively associated with antibodies to the 64 kD fragment, but is also present in 15% of identical twins who remain discordant for diabetes (Christie et al., 1991). In contrast, antibodies to the 40 kD and 37 kD antigens were found in the majority of Type 1 diabetic patients (78%), correlated well with ICA. However no association was found with the 64 kD fragment and the 40 kD and 37 kD autoantibodies were present in only 2% of discordant twins (Genovese et al., 1991). All autoantibodies described so far belong to the immunoglobulin G class and freely cross the placenta. In mothers with Type 1 diabetes, therefore, these autoantibodies are transferred to the foetus (Gleichmann and Bottazzo, 1987). In spite of this, there is no reported evidence of babies born to mothers with Type 1 diabetes having perinatal diabetes. This contrasts, for example, with Graves' disease, in which the placental transfer of

antibodies to thyroid-stimulating hormone receptor produces neonatal thyrotoxicosis (McGregor *et al*., 1984). This finding cannot exclude a pathogenic role of autoantibodies in Type 1 diabetes, because failure to transfer the disease trans-placentally could be due to low levels of autoantibodies in the circulation of the mothers.

On the basis of the available evidence, the precise role of autoantibodies in the pathogenesis of Type 1 diabetes is uncertain and still disputed. Nevertheless, the relatively frequent presence of these autoantibodies in prediabetic individuals may be a useful indication of predisposition to the development of Type 1 diabetes, particularly when they are present in high titre and in combination with one another (Seißler et al., 1992).

1.5 AUTOIMMUNE HEPATITIS, ANOTHER EXAMPLE OF ORGAN-SPECIFIC AUTOIMMUNE DISEASE

1.5.1 INTRODUCTION

Autoimmune hepatitis (AIH) is characterised by the histological picture of chronic aggressive hepatitis and elevated levels of serum immunoglobulins and non-organ- and liver-specific autoantibodies. The involvement of the immune system in the pathogenesis of chronic active hepatitis was suspected shortly after its first description in 1947 (Kelsall *et al*., 1947). Several authors (Waldenström and Blutrotein, 1950; Saint et al., 1953) drew attention to the fact that the affected patients, mainly females, have high serum gammaglobulin levels and that the inflammatory cells characteristically present in portal and peri-portal areas are mostly lymphocytes and plasma cells. These patients were subsequently shown to have high titre of circulating autoantibodies against nuclear components (Bouchier *et al*., 1964), the actin of smooth muscle (Johnson *et al*., 1965), liver/kidney microsomes (Rizzetto *et al*., 1973) or a liver cell cytoplasmic protein called soluble liver antigen (Manns et al., 1987).

Autoimmune hepatitis has been divided according to the serum profiles of autoantibodies detected (Johnson and McFarlane, 1993): Type I AIH is associated with smooth muscle antibody (SMA) with or without antinuclear antibody (ANA), type II AIH is associated with anti-liver/kidney microsomal type 1 (LKM-1) antibody and type 3 AIH is associated with antibodies to soluble liver antigen (SLA) in patients who are seronegative for ANA, SMA and LKM-1. Type 2 AIH has been further divided into type 2a (younger, predominantly female patients without evidence of hepatitis C virus [HCV] infection) and type 2b (older, predominantly male patients with HCV infection) (Johnson and McFarlane, 1993).

Although most of these autoantibodies are non organ-specific and may be found in other diseases (Rizzetto *et al*., 1973), when present at high titres they are important diagnostic markers of AIH.

1.5.2 AUTOANTIBODIES AGAINST LIVER-SPECIFIC MEMBRANE ANTIGENS

Autoantibodies directed against liver-specific antigens in patients with AIH were first described by Meyer zun Büschenfelde et al. in 1968. These antibodies react with a macromolecular antigenic complex called "liver-specific protein" or LSP on the hepatocyte cell membrane (Meyer zum Büschenfelde and Miescher,

1972). More autoantibodies against liver-specific antigens were later detected such as liver membrane antibodies (LMA) (Meyer zum Büschenfelde and Hütteroth, 1979), anti-SLA antibodies (Manns et al., 1987) and anti-asialoglycoprotein receptor (ASGPR) (McFarlane et al., 1986). Anti-ASGPR titres are positively correlated with disease activity (McFarlane et al., 1986). More recently it has been demonstrated in our laboratory that children with AIH have antibodies to liver alcohol dehydrogenase (ADH), which is also a component of the LSP complex (Ma et al., 1993).

The role of these autoantibodies in the pathogenesis and course of the disease is not known, but they are of great diagnostic importance (Manns, 1989).

1.5.3 AETIOLOGY

The aetiology of autoimmune hepatitis is unknown. However, similarity in clinical, biochemical, histological and even serological features can be found in AIH and other conditions, some of which have a recognised aetiology (reviewed by Mieli-Vergani and Vergani, 1993). Chronic aggressive hepatitis is a typical histological picture of AIH and may be seen at various stages of primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC) (El-Shabrawi et al., 1987) and in chronic hepatitis B virus infection (Odievre et al., 1983). The presence of various non organ specific autoantibodies in AIH (i.e. antinuclear antibody, smooth muscle antibody and anti-liver/kidney microsoml antibody) can be found in other conditions, such as primary biliary cirrhosis and malignant disease (Rizzetto et al., 1973; Whitehouse and Holborow, 1971).

Environmental factors such as viruses have been proposed to trigger the autoimmune process in AIH (McFarlane, 1984). Indeed, high titres of antibodies against the measles and rubella viruses have been detected in patients with AIH (Christie et al., 1983; Robertson et al., 1987) suggesting that these viruses might be the trigger of autoimmunity in autoimmune hepatitis. The observation that LKM-1 antibody is associated with infection by the recently described hepatitis C virus (HCV) (Lunel et al., 1992) has provoked great interest amongst hepatologists on the possible viral aetiology of AIH. Early reports of a strong association between anti-HCV and LKM-1 appears to divide LKM-positive AIH into two subgroups: one composed of young patients, usually females with high levels of LKM and negative for anti-HCV, in whom liver damage may be due to a direct autoimmune attack; the other subgroup consisting of older patients, frequently males, with low titre of LKM and positive for anti-HCV, in whom the autoimmune process may be a consequence of the viral disease (Lenzi et al., 1990; Margrin et al., 1991; Todros et al., 1991).

Using immunofluorescence technique, anti LKM-1 antibody stains the cytoplasm of human hepatocytes and proximal renal tubules and has been reported to recognise a 50 kD microsomal protein which has been identified as the cytochrome P4502D6 (CYP2D6) (Alvarez et al., 1985; Zanger et al., 1988; Manns et al., 1989). The demonstration of a partial identity between cytochrome CYP2D6 and HCV has led to the suggestion that HCV may initiate autoimmunity, possibly by molecular mimicry (Manns et al., 1991). It has been recently demonstrated in our laboratory that whilst antibodies producing the characteristic LKM-1 fluorescent pattern can react with CYP2D6, not all LKM-1-positive sera do so, particularly if obtained from patients with chronic HCV infection suggesting that LKM-1 in HCV infection recognises epitopes or antigens different from those targeted in AIH which implies that they represent two distinct groups (Ma et al., 1993; Ma et al., in press).

1.5.4 AUTOIMMUNE HEPATITIS "AN IMMUNE MEDIATED DISEASE"

The clinical onset of AIH is characterised by a variety of cellular and humoral immune changes including production of autoantibodies to hepatocytes, increased number of activated T lymphocytes and alterations in number and function of immunoregulatory T lymphocytes. Some of these immune responses are specific for the liver and this has been taken as evidence that AIH is an autoimmune disease (reviewed by Mieli-Vergani and Vergani, 1993).

1.5.4.1 CELLULAR IMMUNE RESPONSE

Early investigation of cellular immune mechanisms involved in the pathogenesis of AIH, largely used in vitro tests of delayed hypersensitivity to crude liver antigen preparations (Smith et al., 1972; Miller et al., 1972; Lee et al., 1975); or cytotoxicity tests with mixed populations of peripheral blood lymphocytes as effector cells and various allogenic monodispersed hepatocyte preparations (Thompson et al., 1974). The consensus of opinion derived from these studies was that patients with AIH had circulating lymphocytes that were "sensitised" to liver antigens and were able to "kill" the various target cells in vitro and that this was true also of patients with HBV-CLD and some with PBC (reviewed by McFarlane, 1984; and Vento and Eddleston 1987).

Patients with autoimmune hepatitis often have evidence of impaired cell-mediated immunity. This is characterised by total T-cell reduction and an increased ratio of helper to cytotoxic/suppressor T cells (Lobo-Yeo et al., 1987). Using various

in vitro assays, impaired T cell suppressor function has been demonstrated in both adults (Nouri-Aria et al., 1985) and children (Lobo-Yeo et al., 1987) with AIH. Impaired immunoregulation, due to decreased T cell suppressor function, could play an important pathogenetic role in AIH by leading to the persistence of autoimmune reactions. Imbalance of helper T cells which may explain the enhanced immune reactivity typical of the disease has been demonstrated in AIH. Lobo-Yeo et al. (1990B) have shown that levels of activated T lymphocytes expressing surface HLA-DR (mainly of the CD8 phenotype) or IL-2R (mainly of the CD4 phenotype) are elevated in the circulation of children with AIH with active disease compared to those with inactive or other forms of chronic liver disease.

Experiments using subpopulations of T and non-T lymphocytes indicated that cytotoxicity was mediated by non-T lymphocytes (Mieli-Vergani et al., 1979). This observation and the blocking effect of aggregated IgG suggested that cytotoxicity, in AIH, is an antibody-dependent cell-mediated cytotoxicity (ADCC). Studies of the hepatic lymphocyte infiltrate have shown a predominance of B lymphocytes and T helper cells with relatively few cytotoxic/suppressor T cells, whereas cytotoxic/suppressor lymphocytes predominate in areas of necrosis (Eggink et al., 1982; Senaldi et al., 1992).

1.5.4.2 HUMORAL IMMUNE RESPONSE

Autoantibodies have been implicated in the pathogenesis of patients with AIH. They comprise non-organ specific (ANA, SMA and LKM antibodies) and organ specific autoantibodies (LMA, LSP and ASGPR antibodies). Humoral evidence for the involvement of autoreactions in liver disease rely on the finding of circulating autoantibodies that fall into one of the three groups (reviewed by McFarlane, 1991): (1) those that are neither organ nor disease specific, such as ANA and SMA antibodies; (2) those such as AMA and LKM antibodies, that are not directed at liver-specific antigens but subtypes of which are mainly confined to certain liver disorders; and (3) those such as LMA, LSP and ASGPR antibodies, that are more particularly related to the liver but are not necessarily confined to a specific hepatic disorder. Antibodies to the liver cell membrane could be involved in liver-damaging immune reactions either through complement-mediated cytolysis or by the recruitment of cytotoxic, Fc receptor bearing, cells (Vergani et al., 1987b).

Low levels of complement factors have been found in chronic active hepatitis and reported by several authors (Munoz *et al*., 1982, Vergani, 1985). These studies show that both patients with AIH and hepatitis B virus (HBV) related CAH

have low concentrations of C3 and C4, but C3d (a split product of C3) levels were increased only in the HBV group, indicating that activation of complement was restricted to HBV-related CAH. Vergani et al. (1985) reported low serum C4 levels in children with AIH and demonstrated that this is not due to complement activation since none of the patients with low C4 had increased levels of C4d, a fragment which is invariably generated when C4 is broken down during activation of classical pathway (Milgrom et al., 1980). Moreover, parents and siblings of the patients also had low C4 levels, suggesting that low C4 levels in AIH are genetically determined (Vergani et al., 1985). In fact, the C4 phenotypes of children with AIH showed a significantly increased prevalence of null allotypes, particularly at the A locus (C4AQ0). Genetically determined low C4 levels also occur in other autoimmune disorders such as systemic lupus erythematosus (SLE) (Fielder et al., 1983) and Type 1 diabetes (Vergani et al., 1983).

1.5.4 GENETIC PREDISPOSITION

White patients with AIH have an increased frequency of the histocompatibility antigens HLA A1/B8/DR3, a haplotype characteristically associated with other autoimmune conditions, including autoimmune thyroiditis, systemic lupus erythematosus, insulin-dependent diabetes, in which there is excessive production of antibody to extrinsic and auto-antigens (Galbraith et al., 1976). In a reported family with multiple occurrence of AIH, female sex and possession of DR3 was more important than B8 in conferring susceptibility to the disease (Hodges et al., 1991), suggesting that a defective antigen recognition by T cells in the context of class II HLA molecules plays a central role in generating autoimmunity, in association with gender determined factors (genes and hormones). Recently, it has been shown that susceptibility to AIH is conferred independently by two genes within the major histocompatibility complex linked to the DR3 and DR4 genes (Donaldson et al., 1990). When compared to the DR4-positive patients, those with A1/B8/DR3 were shown to present at an earlier age, to relapse more frequently and to be more frequently referred for liver transplantation. Haplotypes conferring susceptibility to the juvenile or mature form of AIH have been further characterised and the possession of the haplotype A1-B8-DRB3*0101-DRB1*0301-DQA1*0501-DQB1*0201-DPB1*0401 has been shown to predispose to the development of the disease in paediatric age (Doherty et al., 1991). This study shows the importance of the DQ alleles in predisposing to AIH.

Children with AIH have an isolated partial deficiency of the complement

component C4, which is not due to impaired hepatic synthesis or immune consumption, but is genetically determined and is associated with the possession of a silent gene at the C4A locus, C4AQ0 (Vergani et al., 1985). C4AQ0 either in linkage with A1/B8/DR3 or on its own has been reported in association with other autoimmune disorders (Lachmann and Peters, 1982) and could represent the true disease susceptibility gene. The genetically determined partial C4 deficiency resulting from the possession of null C4 genes may be a predisposing factor to autoimmunity. C4 has a key role in virus neutralisation (Daniels et al., 1969) and failure to eliminate viruses might lead to the development of immune and autoimmune reactions directed against antigens on the persistently infected cells. The specificity of the autoimmune reaction would possibly derive from the tropism of the virus.

All the above data suggest that AIH conforms to a multi-factorial model (Whittingham et al., 1981). This proposes that a disease develops when a threshold is exceeded by a number of different combinations or interactions of multiple genetic and environmental factors.

CHAPTER 2

INTRODUCTION TO THE PRESENT STUDY

2.1 INTRODUCTION

Development of insulin dependent diabetes, as discussed previously, involves both a genetic and an environmental component. The role of the immune system is to respond to stimuli coming from the environment under the control of genetic factors. It has been suggested that the immune attack on the β-cell has both cellular and humoral autoaggressive elements (reviewed by Vergani, 1987a). T lymphocytes are capable of killing the insulin-producing ß-cells both directly and indirectly, through the recruitment of other effector mechanisms.

Figure 2.1 summarizes the possible mechanisms leading to an immune attack on the ß-cell. A ß-cell surface antigen is presented to helper T (T_H1 or T_H2) lymphocytes either directly on the cells's surface or by an antigen presenting cell (APC), in the context of class II MHC antigens. Then if T suppressor (Ts) cells do not oppose, the T helper cells alert one or more effector cell mechanisms. Direct killing of the ß-cell can derive from activation of cytotoxic T lymphocytes (Tc) which, through their receptors, recognise target antigens in association with class I MHC antigens on the cell surface. The ß-cell can also succumb to the joint attack of T_H1 and activated macrophages. This pathway operates mainly through the release of cytokines produced by macrophage and T_H1. Macrophage-derived cytokines such as tumour necrosis factor (TNF) and interleukins (IL) 1 have the potential to kill the ß-cell in their own right (Mandrup-Poulsen et al., 1987a; Sandler et al., 1991; Ling et al., 1993). T_H1-derived cytokines can attract macrophages to the site of reaction with the target cell, and make them metabolically 'angry'. Angry macrophages can dispose of ß-cells by the release of monokines, proteolysis and phagocytosis. B-lymphocytes can also be engaged in an immune attack on the ß-cells under effect of T_H2-derived cytokines by producing autoantibodies. A target coated with an antibody can be destroyed by activation of the complement system or by killer cells (Roitt, 1988). In the context of the present study, it is important to note the potential key role played by soluble factors - cytokines - in both the orchestration and execution of the immune attack on the ß-cell.

This chapter will give a brief biological definition for some of cytokines which are likely to play an important role in the pathogenesis of Type 1 diabetes.

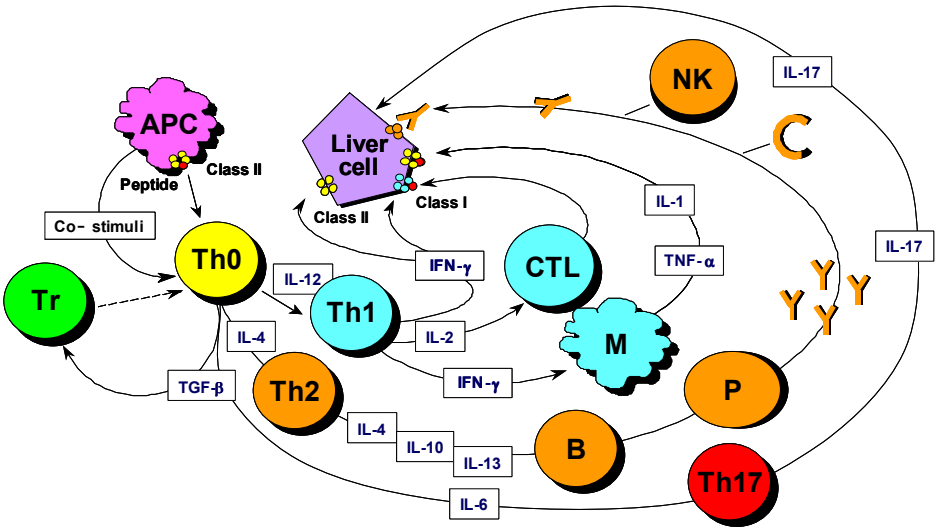


Figure 2.1. ß-cell immune attack. A ß-cell ("target cell") antigen is presented to helper (T_H1 or T_H2) lymphocytes either directly on the cell's surface or by an antigen presenting cell (APC), in the context of class II MHC antigens. If there is no opposition from T regulatory (Tr) lymphocytes, then a variety of effector mechanisms are triggered. ß-cell destruction could derive from the action of: (a) T cytotoxic (CTL) lymphocytes which react with the self antigen(s) in the context of class I MHC antigens or (b) cytokines produced by T_H1 lymphocytes and recruited macrophages. K, killer lymphocytes; B, B lymphocyte; M, macrophage; P, plasma cell.

2.2 CYTOKINES

"Cytokine" is one term for a group of protein cell regulators, variously called lymphokines, monokines, interleukins and interferons, which are produced by a variety of cells in the body. Since most cytokines possess more than one biologic property, descriptive names can be misleading. Therefore, a nomenclature has been developed that employs the term "interleukin" followed by a number (Aarden et al., 1979).

Cytokines generally are glycoproteins with molecular weights of less than 80 kD. They are involved in immunity and inflammation where they regulate the amplitude and duration of the immune response. Cytokines are extremely potent, generally acting at picomolar concentrations. They interact with high affinity cell surface receptors specific for each cytokine group (Reviewed by Balkwill and Burke, 1989).

Cytokines have been reported to be involved in the immunopathology of several autoimmune diseases including Type 1 diabetes (Dinarello and Mier, 1987). There is evidence that cytokines could have a direct role in β-cell death (Mandrup-Poulsen et al., 1989). The macrophage released cytokines, TNF-α and IL-1, are cytotoxic to islet β-cells in vitro (Mandrup-Poulsen et al., 1986; Campbell et al., 1988). Interleukin-6, also produced by macrophages, is a key mediator of multiple inflammatory and immune responses (Hirano et al., 1990), and regulates insulin secretion in vitro in concert with IL-1 (Campbell et al., 1989). IFN-γ, produced by activated T lymphocytes, activates macrophages (Murray, 1988), enhances class I Major Histocompatibility Complex (MHC) antigen expression and induces class II expression in combination with TNF on normal cultured human islet cells (Pujol-Borrell et al., 1987); IFN-γ also enhances TNF induced human islet cells cytotoxicity (Soldevila et al., 1991). These studies indicate that cytokines may have a role in the pathogenesis of Type 1 diabetes.

The biological properties of these cytokines and their potential role in the pathogenesis of Type 1 diabetes will be discussed next.

2.2.1 INTERLEUKIN-1

The first described activity of IL-1 was in 1972 by Gery et al. They discovered a lymphocyte-activating factor (LAF) in the supernatant of cultures of adherent human peripheral blood cells and murine splenocytes, which was mitogenic for murine thymocytes. In 1974, it was reported that cultured human monocytes also secreted a B cell-activating factor (BAF) that stimulated antibody

production by T cell-depleted murine splenocytes (Wood and Gaul, 1974). Subsequent analyses revealed that the biochemical properties of LAF and BAF were similar, resembling the structure of a pyrogenic macrophage-derived factor which was later called IL-1.

IL-1 is a 12-16 kD glycoprotein. It is produced by all the macrophage-containing tissues including pulmonary alveolar cells, hepatic Kupffer cells and bone marrow (Moore et al., 1980). Resting monocytes or macrophages produce little or no IL-1. Mitogens such as lipopolysaccharide (LPS) and phorbol myristic acetate (PMA) were reported to stimulate macrophages to induce IL-1 production (Oppenheim et al., 1979). Activated lymphocytes can also stimulate macrophages to produce IL-1 by releasing lymphokines such as colony-stimulating factor, which stimulate macrophages (Moore et al., 1980). IL-1 exists in 2 molecular forms called IL-1α and IL-1β (reviewed by Dower and Urdal, 1986). Both peptides although distinct gene products, share many biological activities and are recognised by the same receptors (Wood et al., 1985; Kilian et al., 1986). Biologically active IL-1α, but not IL-1β, has been detected on the surface of cells and can thus participate in interactions during cell-to-cell contact. Therefore after its production, IL-1α remains mainly cell-associated, whereas IL-1β is primarily the released form.

IL-1 has wide-ranging biological effects and plays a central role in the activation of cells involved in inflammatory and immune responses (reviewed by Pober and Cotran, 1990; Rees, 1992). IL-1 can stimulate proliferation of mouse and human T lymphocytes (Mizel et al., 1981), possibly due to the ability of IL-1 to induce the release of IL-2 (Smith et al., 1980). Production of immunoglobulins by human B lymphocytes also appears to be augmented by IL-1. Human IL-1 can partially restore pokeweed mitogen (PWM)-induced immunoglobulin production in cultures of macrophage-depleted human lymphocytes (Rosenberg and Lipsky, 1981). Some of its effects on B cells may be mediated through its induction of IL-6, which is a B cell differentiation factor (Dinarello et al., 1987).

IL-1 also regulates several non-immunological responses involving macrophages. It stimulates the release of acute phase reactants by hepatocytes, the release of prostaglandins and collagenase from synovial cells, and the cell division of fibroblasts. Therefore, IL-1 may be involved in fibrosis-associated with cell-mediated immunological reactions such as rheumatoid arthritis as well as in non-immunological inflammatory reactions (reviewed by Mantovani and Dejana, 1989)

Naturally-occurring inhibitors of IL-1 have been reported in a variety of biological fluids such as serum, synovial exudates and urine (Larrick, 1989;

Seckinger and Dayer, 1987). Cultured mononuclear cells from synovial fluid have been formed to secrete an IL-1 inhibitor, which was identified as an IL-1 receptor antagonist (IL-1ra) (Roux-Lombard et al., 1992). IL-1ra blocks binding of IL-1 to its receptors and has been cloned and produced in recombinant form and found to reduce the severity of various diseases such as sepsis, colitis, arthritis and diabetes in animal models (reviewed by Dinarello and Thompson, 1991). There are two IL-1 receptors, now indicated as IL-1R type I and type II, corresponding to the 80 kD (Sims et al., 1988) and 68 kD (Horuk and McCubrey, 1989) IL-1 binding proteins on T and B cells respectively. Although the type I receptor appears to account for nearly all of the biological activity of IL-1, the two receptors may cooperate in binding and signal transduction on some cells (reviewed by Dinarello and Thompson, 1991). The type II receptor is probably proteolytically cleaved to yield the soluble IL-1 binding protein described by Symons et al. (1991). This molecule is released as a 47 kD protein from Raji cells, and has high affinity for IL-1β (but does not bind IL-1α), which suggests that it is a naturally-occurring inhibitor for IL-1β (Scapigliati et al, 1989; Scott *et al*., 1991).

2.2.2 INTERLEUKIN 2 (IL-2)

Interleukin 2 was originally called T cell growth factor (TCGF) and described as a lymphokine present in lymphocyte-conditioned medium that enhanced thymocyte mitogenesis and maintained in vitro growth of T-cell lines (Morgan et al., 1976). IL-2 is 15 kD glycoprotein. Studies of isolated subpopulations of lymphocytes and thymocytes show that antigens induce IL-2 production from CD4 helper T cells. Potent polyclonal mitogens and class I MHC alloantigens can also stimulate the CD8 T lymphocytes to produce IL-2 (Smith, 1987). In addition, a subset of large granular lymphocytes which are closely related to NK cells can release IL-2 when stimulated with mitogens such as PHA (Itoh et al., 1985).

In order to exert its biological effects, IL-2 must interact with specific membrane receptors (Robb et al., 1981). This interaction is critical to the development of a normal immune response. Interleukin 2 receptors are not expressed on resting T lymphocytes, but are expressed rapidly following activation with antigen or mitogen. The IL-2 receptor complex comprises an α chain of 55 kD (also known as CD25, Tac, p55), first identified in an adult T cell leukaemia (Smith, 1987), and a ß chain of 75 kD (p75) (Greene and Leonard, 1986). Depending on the relative expression of the α and β subunits at the cell surface, they constitute low (K_d 10^{-8}M), intermediate ((K_d 10^{-9}M), or high affinity (K_d 10^{-11}M) receptor for IL-2

(Hatakeyma et al., 1989). Controversy has surrounded the presence of a γ chain in the receptor complex: the question has recently been settled following the cloning of a γ chain from a human T cell line into which the ß chain had been transfected (Takeshita et al., 1992). The minimal receptor configuration for signal transduction now appears to be the IL-2Rßγ heterodimer, which binds its ligand with an intermediate affinity, whilst the combination of α, ß and γ chains results in a high affinity binding. The γ chain also appears to be critical for internalisation of the receptor-ligand complex (Takeshita et al., 1992). The α chain alone binds IL-2 with a relatively low affinity and lacks the ability to transduce an activation signal. However, IL-2α chains have a slow turnover rate on the cell surface (>6 hours compared with 2-3 hours for the IL-2Rαß heterodimer, or 15 minutes when bound to IL-2) and act as a trap for IL-2 which can then be passed to intermediate or high affinity receptors, by lateral diffusion, for internalisation (Saragovi and Malek, 1990). Such a process enhances the overall affinity of a cell for IL-2, shifting the dose-response curve to the left and increasing the sensitivity of IL-2 mediated activation.

The biological effects of IL-2, although originally defined on the basis of the growth of T cells in culture, have expanded to include interaction with macrophages, activated B cells (Mond et al., 1985, Kishi et al., 1985), natural killer (NK) (Ortaldo et al., 1984) and other cytotoxic cells (Gillis et al., 1979). This response to IL-2 requires the expression of α or β IL-2-binding protein on the unstimulated mononuclear cells. Monocytes and macrophages normally express a low density of IL-2R α chain. Activation of macrophages by IFN-γ or LPS, however, results in the development of high affinity IL-2 receptors consisting of p75 and the p55 Tac antigen. Addition of IL-2 to these activated macrophages results in the activation of the Tac, granulocyte macrophage colony-stimulating factor (GM-CSF), and G-CSF genes and also augments the tumoricidal activities of these macrophages.

Large granular lymphocytes (LGL), which exhibit antigen-nonspecific NK cell activity express the p75 antigen; after in vitro incubation with IL-2, LGL begin to express both p55 Tac antigen and the high-affinity IL-2 receptor complex. When activated, these cells have been shown to mediate potent antitumour cytotoxicity in vitro against target tumour cells freshly derived from "solid" tumour tissue (Rosenberg et al., 1985).

High-affinity receptors for IL-2 have been found on B lymphoblasts. Activated normal as well some transformed B lymphoblasts, but not resting B cells, express about 30% as many Tac antigen receptors for IL-2 as do activated T cells.

IL-2 can induce both increased antibody production and proliferation (Falkoff et al., 1984).

Lymphocytes exposed to IL-2 also release a number of cytokines. These include IFN-γ (Kasahar et al., 1983), TNF-α and β (Nedwin et al., 1985) and differentiation factors such as IL-4 and IL-6 (Ohara and Paul, 1987; Rees, 1992).

One of the important aspects of T cell-derived cytokines is that many act in synergy with others, and thus the net effect may be considerably greater than that of the individual components. Well known synergies are those of IL-2 and IL-4 in T cell growth (Spits et al., 1987). IL-10 was also found to synergise with IL-2 for the proliferation of mature and immature T cells (MacNeil et al., 1990), their differentiation into cytotoxic effectors (Chen and Zlotnik, 1991) and more recently was shown to enhance the proliferation and differentiation of normal and leukaemic B cells upon CD40 triggering (Anne-Catherine et al., 1993).

2.2.3 INTERLEUKIN 4

Interleukin 4 (IL-4) is another T-cell-derived cytokine. It is similar in size to IL-2 but binds to a distinct cell surface receptor (Ohara and Paul, 1987). IL-4 is an alternative (to IL-2) growth factor for certain T cells but exerts its principal action on B-cells where it serves both as an activation factor for resting cells and as an isotype switching factor to IgG1 and immunoglobulin E for stimulated cells (Snapper *et al*., 1988). IL-4 acts also on macrophages as an activating factor by inducing MHC Class II molecules and induces proliferation of mouse mast cells especially in synergy with IL-3 (Paul and Ohara, 1987). According to Mosmann et al. (1986), two distinct cytokine secretion patterns have been defined among a panel of mouse T cell clones, T_H1 and T_H2. T_H1 cells secrete predominantly IL-2 and IFN-γ, and are associated with delayed-type hypersensitivity reactions while T_H2 cells secrete IL-4, IL-5, IL-6 and IL-10 and are associated with high antibody levels (Mosmann et al., 1986).

The effect of IL-4 on the kinetics of other cytokines has been studied in further experiments and it has been shown that IL-4 suppresses LPS-induced stimulation of IL-1, TNF-α and IL-6 synthesis by human monocytes, the effect being manifested at the level of mRNA (Cheung et al., 1990). The addition of IL-4 to activated $CD4^+$ cell cultures induces secretion of high levels of T_H2 cytokines (Seder et al., 1992) and preferentially stimulates the growth of T_H2-like cells (Fernandez-Botran et al., 1988) while IFN-γ, which is produced by T_H1 cells, was found to inhibit IL-4-induced switching. Recently, it was demonstrated that the combination of IL-4 and IL-10 increased T cell replication but results in lower immunoglobulin

production (Rousset et al., 1992). Many cell types bear receptors for IL-4 such as endothelial cells and certain tumour cell lines (Lowenthal et al., 1988), which renders these cells susceptible to the effects of IL-4.

2.2.4 INTERLEUKIN 6

Interleukin 6 (IL-6) was originally identified as a T cell-derived factor acting on B cells, in order to induce the final maturation to antibody-forming plasma cells and called B cell stimulating factor-2 (BSF-2) (Yoshizaki et al., 1982). IL-6 is a 26 kD glycoprotein. It can be produced by several cells, including T and B lymphocytes, monocytes, endothelial cells and fibroblasts (Aarden and Van Kooten, 1992). IL-6 appears to function as a growth factor for certain transformed B cells. The identification of IL-6 as plasmacytoma and hybridoma growth factor supports this idea (Kawano et al., 1988). IL-6 also promotes the proliferation of Epstein-Barr virus-infected B cells and permits their growth at low densities (Tosato et al., 1988). It has been demonstrated that freshly isolated human myeloma cells both produce IL-6 and express IL-6 receptors (Kawano et al., 1988). Moreover, anti-IL-6 antibodies inhibit the growth of the myeloma cells in vitro, suggesting that IL-6 may function as an autocrine signal in the process of uncontrolled proliferation during myeloma (Kawano et al., 1988). A relationship between deregulated IL-6 production and polyclonal plasmacytosis has been observed in patients with uterine cervical carcinoma, rheumatoid arthritis, and acquired immune deficiency syndrome (AIDS) (Kishimoto and Hiravot, 1988).

IL-6 can act as a stimulatory factor for T cells at different stages of differentiation. For example, IL-6 can stimulate the proliferation of $CD4^+$ and $CD8^+$ subsets of thymocytes that have been co-stimulated with IL-2 and IL-4 (Hodgkin et al., 1988). In mature T cells, IL-6 can activate the production of IL-2, although it does not stimulate cellular proliferation (Garman et al., 1987).

IL-6 acts as a hepatocyte-stimulating factor (HSF), inducing the synthesis of acute phase proteins in the liver, including C-reactive protein (CRP), α1-acid glycoprotein, fibrinogen, and α-2-macroglobulin (Revel, 1988; Richards *et al*., 1991). The expression of some of these acute-phase proteins is also modulated by IL-1 or TNF-α, which combine synergistically with the HSF activity of IL-6 (Wong and Clark, 1988). High doses of IL-6 also produce fever by stimulating the hypothalamic fever centre; IL-6 therefore acts as an endogenous pyrogen (Frei et al., 1989).

IL-6 can be induced in response to other cytokines such as recombinant

tumour necrosis factor (Zhang et al., 1988) and IL-1 (Hirano and Kishimoto, 1989). High levels of circulating IL-6 have been found in several diseases, including fulminant liver failure and chronic hepatitis B (Sheron et al., 1990). In each case high levels of IL-6 have been found to correlate with similar elevated high levels of TNF-α, implying either that both are released as a result of a common stimulus or that circulating IL-6 is produced in response to circulating TNF or other cytokines (Sheron et al., 1990).

2.2.5 INTERLEUKIN-10

Interleukin-10 (IL-10) (originally known as cytokine synthesis inhibitory factor (CSIF)) is an acid-labile 35-40 kD homodimer that is produced by T_H2 but not T_H1 cell (Mossmann et al., 1986). It was discovered and isolated on the basis of its ability to suppress cytokine synthesis by T_H1 helper T cell (e.g. IL-2/IFN-γ) (Fiorentino et al., 1989; Moore et al., 1990; Vieira et al., 1991). IL-10 suppresses both cytokine production and antigen-specific proliferation of cultured clones of T_H1 cells when they are activated in an accessory cell-dependent manner (Vieira et al., 1991). The addition of anti-IL-10 antibodies to primary cultures of helper T cells, in some in vitro systems, enhances the emergence of cells bearing a T_H1 cell phenotype suggesting that IL-10 may play a role in diminishing delayed-type hypersensitivity reactions and other T_H1 cell-mediated responses (Howard and O'Garra, 1992).

IL-10 also suppresses the ability of murine macrophages, but not B cells, to stimulate T_H1-cell clones to synthesize cytokines (Fiorentino et al., 1991). While IL-10 is a potent immunosuppressant of macrophage function, it exerts a wide array of immunostimulatory effects on B cells. These include enhancement of in vitro viability and upregulation of MHC class II molecule expression by highly purified, small, dense B cells obtained from spleens of unstimulated mice (Go et al., 1990). The effects of IL-10 on activated B cells have been studied in vitro. Rousset et al. (1993) and Defrance et al. (1992) reported that IL-10 augments both proliferation and differentiation of activated B cells into antibody-secreting cells and this effect was enhanced in the presence of IL-2 (Anne-Catherine et al., 1993). In addition, IL-10 was found to be a stimulant to other cell types. It augments the proliferative response of murine thymocytes and T cells that have been activated with IL-2 and IL-4 (Suda et al., 1990; MacNeil et al., 1990), and may, therefore, play a role in T-cell development. The ability of IL-10 to suppress synthesis of a subset of T-cell-derived cytokines (e.g. IFN-γ, IL-2 and TNF-β), in addition to suppression of monokine synthesis, makes IL-10 an attractive candidate for prolonging allograft

survival, and for treatment of a variety of T-cell-mediated autoimmune diseases, such as Type 1 diabetes and multiple sclerosis (Howard and O'Garra, 1992).

2.2.6 INTERFERON-γ

Interferon-γ was originally identified by its antiviral activity and was discriminated from IFN-α and IFN-β by its lability at pH 2.0, a property often used as a simple method of identification (Faltynek and Kung, 1988).

IFN-γ is a 17 kD glycoprotein. It is produced mainly by activated T lymphocytes following exposure to antigen or mitogen (Street and Mosmann, 1991). Using mouse T cell clones, it has been found that T_H1 but not T_H2 cells secrete IFN-γ (Mosmann et al., 1986). Natural killer (NK) cells also produce IFN-γ when triggered by mitogens such as phytoheamoagglutinine (PHA) (Matsumura and Nakano, 1988). Production of IFN-γ by T lymphocytes can be dependent upon secretion of other cytokines. Farrar et al. (1981) found that IFN-γ production by lymphocytes is dependent upon secretion of IL-1 by accessory adherent cells and secretion of IL-2 by T helper cells. IL-2 can also stimulate the production of high titres of IFN-γ in culture in the absence of any other known inducers (Lee et al., 1986).

IFN-γ not only acts as an antiviral agent but also exerts several effects on vital cellular and body functions, including cell proliferation, hormone stimulation, immune responses, and tumour development (reviewed by Ijzermans and Marquet, 1989; Billian and Dijkmans, 1990). The immunomodulatory activities of interferon are mediated by its effects on the cells responsible for host defense, i.e., macrophages, T and B lymphocytes and LGL with NK activity. IFN-γ exerts effects on macrophages, and acts as a potent macrophage activating factor, resulting in increased production of IL-1α (Arenzana-Seisdedos et al., 1985) and TNF-α (Collart et al., 1986). Activation of macrophages is accompanied by increased expression of receptors for the Fc portion of immunoglobulins (FcR) (Vil_ek and Demaeyer, 1984). This results in the increase in both the phagocytosis of immune complexes and in the capacity of the macrophages to lyse antibody-coated bacteria, parasites, and tumour cells by antibody dependent cell-mediated cytotoxicity (ADCC). IFN-γ boosts and maintains the level of class II histocompatibility molecules on the surface of macrophages, (Kelley et al., 1984) as well as on other cell types such as T and B cells (Pober et al., 1983; Berrih et al., 1985). IFN-γ induces expression of class II MHC antigens on epithelial, endothelial and connective tissue cells, lymphoid cells and monocyte cell lines (Steeg et al., 1982, Wong et al., 1983) and of class I MHC

on cytotoxic T lymphocyte targets (Pestka et al., 1987). The expression of class II will allow these cells to become active in antigen presentation.

IFN-γ can either augment or suppress cellular and humoral immunity, depending on the dose and time of administration (Trinchieri and Perussia, 1985). In general, in vivo administration of IFN-γ before or concomitant with antigenic sensitization has considerable inhibitory effects, whereas administration of interferon after antigenic sensitization augments both cellular and humoral immune responses (Baron *et al*., 1991). Anti-proliferative effects of IFN-γ in immune regulation have been studied in vitro using murine helper T lymphocyte clones and found that IFN-γ inhibits the proliferation of T_H2 and favours development of T_H1, suggesting the ability of IFN-γ to direct T_H1-like and T_H2 immune responses (Gajewski *et al*., 1989; Noble et al., 1993). IFN-γ also acts as a B-cell maturation factor, inducing the maturation of resting B lymphocytes and resulting in surface phenotype changes and immunoglobulin secretion (Leisbon *et al*., 1984). In the mixed lymphocytes reaction (MLR), IFN-γ has been reported to be the predominant cytokine in culture and was responsible for generation of cytotoxic T lymphocytes (CTLs) (Simon et al., 1979). Effects of IFN-γ on natural killer (NK) cell activity has been reported extensively (Trinchieri and Perussia, 1985). NK cell activity is mediated by the cytotoxic effects of large granule lymphocytes, in the absence of prior sensitization, against virus-infected cells or certain tumour cell lines. Both in vivo and in vitro adminstration of IFN-γ enhances the NK cell activities of LGL (Trinchieri and Perussia, 1985).

IFN-γ also affects the complement system, by enhancing production of complement factors (B, C2 and C1-inhibitor) (Littman et al., 1989) and promotes adherence of macrophages to membrane glycoproteins (Show and Mercurio, 1989).

IFN-γ can act synergistically with other cytokines. The effects of this synergism depend mainly on the target cells (reviewed by Balkwill and Burke, 1989). For instance, IL-4 and IFN-γ have similar or synergistic activities, such as activation of T cells, but their actions on immunoglobulin isotype selection or MHC class II expression are differential or antagonistic (Siegel, 1988). IFN-γ acts synergistically with TNF-α to increase class I and II MHC antigen expression on a variety of cells (Baron et al., 1991) and this expression is thought to play an important role in the initiation of the autoimmune process.

2.2.7 TUMOUR NECROSIS FACTOR-α (TNF-α/CACHECTIN)

TNF-α was first found in serum of animals that were primed with

mycobacterial antigens, Bacillus-Calmette-Guérin, and then challenged with lipopolysaccaride (LPS). TNF-α activity caused the necrosis of certain tumours when injected into tumour-bearing animals. This selective killing in vivo occurs without any apparent species-specificity (reviewed by Cerami and Beutler, 1988). A molecule identical to TNF-α and named cachectin (it cause cachexia) was isolated from serum of endotoxin-sensitive mice treated with LPS (Beutler et al., 1985).

TNF-α is a 17 kD polypeptide (Beutler et al., 1985; Spies et al., 1986). Many cell types can produce TNF-α. Monocyte-macrophages produce TNF-α in response to stimulation by phorbol myristate acetate, lipopolysaccharides and bacillus calmette Guérin (Männel et al., 1980; Matthews, 1981). Other cells, including T and NK cells can produce TNF-α (Feldmann et al., 1989). A number of endogenous mediators such as IL-1, IL-3, granulocyte macrophage colony stimulating factor (GM-CSF), IFN-γ were reported to induce production of TNF in conjunction with LPS and TNF-α itself (Oppenheim et al., 1989). A related molecule, lymphotoxin, produced by activated lymphocytes, shares many of IL-1 properties (Tartaglia and Goeddel, 1992). It binds to the same receptor as cachectin/TNF-α and evokes similar biological responses (Paul and Ruddle, 1989).

TNF-α has a variety of immunological properties. Like IL-1, TNF-α is co-mitogenic for thymocytes and enhances IL-2 receptor and MHC class II expression on T cells (Feldmann et al., 1989). In addition, TNF-α has been shown to augment B cell proliferation, surface immunoglobulin receptor expression, and antibody production (Beutler and Cerami, 1989). TNF-α may play a critical role in preventing the spread of invading pathogens by stimulating release of other cytokines from lymphocytes. The spread of invading pathogens has been shown to be reduced significantly following administration of small amounts of TNF-α in mice infected with murine malaria (Djeu et al., 1986). TNF-α is a mediator of general inflammation, it induces (together with IL-1) a fever by the stimulation of hypothalamic prostaglandin-E_2. It also stimulates the synthesis of IL-1, IL-6, IL-8 and several other pro-inflammatory mediators (Bachwich et al., 1986). TNF-α promotes the expression of adhesion molecules on leucocytes and endothelial cells. This mechanism initiates the recruitment of leucocytes to the site of infection.

TNF-α acts synergistically with IFN-γ, and causes enhanced anti-proliferative activity which is known to decrease the growth of cancer cells in culture (Brouckaert et al., 1986); expression of class I and II MHC antigens is also induced on various cell types (Lapiérre et al., 1988, Leeuwenberg et al., 1988). The

cytokine-induced expression of MHC antigens on various cells during an immune response have important immunological implications and suggest an immunoregulatory role for cytokines.

2.3 CYTOKINES AND AUTOIMMUNITY

A role for cytokines in the initiation of the autoimmune process has been proposed for several autoimmune disorders (reviewed by Brennan and Feldmann, 1992). Increased levels of IL-1, IL-2 and IL-6 have been found in the synovial fluid of rheumatoid arthritis (RA) and some of these cytokines are correlated with disease activity (Hopkins et al., 1988; Buchan et al., 1988; Feldmann, 1988). Lymphoid cells from the thyroid glands of patients with autoimmune disease were found to produce spontaneously cytokines such as IFN-γ, TNF-α, lymphotoxin, IL-6, IL-1α and IL-1β (Grubeck-Lobenstein et al., 1989). High levels of cytokines were also observed in the plasma of patients with systemic lupus erythematosus and chronic progressive multiple sclerosis (Huang et al., 1988; Cavallo *et al*., 1994). Most experimental data indicate that cytokines have direct effects not only on the state of immune activation in autoimmune diseases but also on the target organ which is important in the autoimmune disease. Cytokines such as IL-1 (both α and β) and TNF-α were found to be cytotoxic to several cell lines including β-cells. They also activate osteoclasts involved in bone resorption, and bone synthesis is inhibited by both IL-1α and TNF-α (reviewed by Akira et al., 1990). In addition, IFN-γ is capable of inducing aberrant class II MHC antigens on a variety of cell lines such as thyroid cells, β-cells and hepatocytes (reviewed by Cavallo *et al*., 1994). Such expression is thought to be important and rendering target cells more susceptible to immune attack and to the cytotoxic effect of other cytokines.

2.3.1 'ABERRANT' MHC EXPRESSION INDUCED BY CYTOKINES

The role of 'aberrant' expression of MHC class II molecules and antigen presentation in the induction of endocrine autoimmunity was initially proposed by Bottazzo et al. (1983). If cells are induced to express MHC class II antigens, they may then 'present' their particular cell surface antigen to potentially autoreactive T helper lymphocytes, which would initiate an autoimmune response. The hypothesis is important since there is a marked genetic component to the risk of acquiring the disease which is linked to class II MHC genes (HLA-DR type).

In some autoimmune states, inappropriate expression of class II antigens has

been noted on the target organ. This was observed in the synovia of joints affected with rheumatoid arthritis (Klareskog et al., 1982), thyrocytes in Hashimoto's and Graves' diseases (Hanafusa et al., 1983) and also on the islet β-cells of both patients and animals with Type 1 diabetes (Bottazzo et al., 1985, Dean et al., 1985). This has led to the proposition that such 'aberrant' class II expression enables these cells to present tissue bound self antigen and initiates the disease process.

The expression of class II MHC products, on β-cells, was initially observed by Bottazzo (1985) in a single case of recent onset of Type 1 diabetes. More cases have been studied by Foulis et al. (1989). They reported that 21 out of 23 patients with recent disease had aberrant expression of class II MHC molecules on β-cells. No such expression was seen on glucagon-secreting α cells or somatostatin secreting δ cells. In addition, Foulis found a marked hyper-expression of class I MHC molecules on islet endocrine cells. No such class I or II MHC expression was found in the pancreas of patients with Type 2 diabetes, chronic pancreatitis or cystic fibrosis. Cytokines are probably the principal modulating influence on MHC product expression in vitro and in vivo. The ability of interferons to induce MHC-product expression was first shown by Lindal and co-workers (1973; 1976). They showed that interferons induce histocompatibility antigen expression on mouse L1210 lymphoma cells in vitro and on lymphoid cells in vivo. The effect has been demonstrated with both natural and recombinant interferons, and of type I (α and β) and type II (γ). The most striking effects, however, were demonstrated with IFN-γ which enhances the expression of class II antigens or induces them de novo in a large number of cell types such as human and animal β-cells.

Induction of MHC expression on human and murine β-cells by IFN-γ has been reported by several authors (Wright et al., 1986; Campbell et al., 1986; Pujol-Borrell et al., 1987). They found initially that IFN-γ enhances the expression of HLA-A,B,C (class I MHC) but not HLA-DR (class II MHC) on human pancreatic β-cells in vitro. Induction of class II MHC antigens was observed on human islet cells only when exposed to the combination of IFN-γ plus TNF-α (Pujol-Borrell et al., 1987). Recently, Soldevila et al. (1991) reported that the combination of IFN-γ plus TNF-α not only induced expression of class II antigens but was also associated with their cytotoxic effects on human islet cells in vitro. This observation together with the initial report of aberrant expression of class II and the recent finding of IFN-γ producing cells in the pancreas of newly-diagnosed diabetic patients (Foulis et al., 1991) is of potential interest because a role for these cytokines in the destruction of β-cells is suggested.

2.3.2 CYTOTOXICITY OF CYTOKINES

All cytokines apparently have a growth factor activity, but the ability to inhibit cell growth or kill cells is limited to a number of them such as TNF-α, IL-1 (α and β) and IFN-γ (Mestan et al., 1986). These are cytotoxic to a range of cell types including β-cells (Mandrup-Poulsen et al., 1989). IL-1β, TNF-α and IFN-γ, alone or in combination have been reported to be cytotoxic to murine and human islet β-cells. IL-1β causes selective morphological and functional changes to pancreatic β-cells in vitro suggestive of cytotoxicity (Bendtzen et al., 1986; Mandrup-Poulsen et al., 1987a; Sandler et al., 1987). In addition, IL-1β has been shown to have complex dual effects on β-cell function: at a low concentration and short exposure (60-90 min), IL-1β stimulates proinsulin biosynthesis and insulin secretion while at a high concentration and long (>24 hour) exposure, it causes inhibition of proinsulin biosynthesis and insulin secretion (Spinas et al., 1987). TNF-α alone, or in combination with IFN-γ was also found to exert cytotoxic effects on rat and mouse islet cells in vitro, resulting in a reduction of insulin release and with obvious β-cell damage (Campbell et al., 1988). The effect of cytokines on human islets has been reported recently by Soldevila and his colleagues (1991). These authors found that combination of IFN-γ and TNF-α exerts a cytotoxic effect on human islet cells in vitro with a reduction in insulin release, and associated cell damage. TNF-α alone produced inhibition of insulin release while IFN-γ had little effect. In addition, they observed that there is a time course relationship between MHC class II induction and the cytotoxic effect of IFN-γ plus TNF-α on the same islet cells. The synergistic effects of combination of IFN-γ and TNF-α have been reported in a variety of situations, including induction of MHC molecule expression (Pujol-Borrell et al., 1987), protection of cells from viral infection (Wong and Goeddel, 1986) and cytotoxicity to tumoral cell lines (Sugarman et al., 1985). It has been postulated that this synergism is due to the up-regulation of TNF-α receptors on the cell surface by IFN-γ (Aggarwal et al., 1985).

2.3.3 CYTOKINE PROFILE

Two types of helper T cell clones (T_H1 and T_H2) have been recognized in the mouse on the basis of a pattern of cytokine secretion (Mosmann et al., 1986). T-cell clones of the T_H1 subset synthesize and secrete IL-2 and IFN-γ, whereas T-cell clones of the T_H2 subset produce IL-4, IL-5, IL-6 and IL-10 but not IL-2 and IFN-γ. Human $CD4^+$ T cell clones with an intermediate cytokine profile have also been

described and termed T_H0 (Romagnani, 1992). It was thought that this classification is not valid for human T cells until recently. It has been found that $CD4^+$ T-cell clones that exhibit T_H1- or T_H2-like profiles can accumulate in tissue or peripheral blood of patients in different disease states (Romagnani, 1992). These clones were found also to differ in their cytolytic potential and mode of help for B-cell Ig synthesis. For instance, the majority of T_H1 (77%), but only a minority of T_H2 (18%) clones exhibit cytolytic activity in a 4h PHA-dependent assay, while T_H2 (noncytolytic) clones induced synthesis of IgM, IgG, IgA and IgE by autologous B cells in the presence of the specific antigen and the degree of response was proportional to the number of T_H2 cells added to B cells (Romagnani, 1992). Based on these findings, it has been suggested that the T_H1 subset represents the most important effector cells in inflammatory reactions associated with vigorous delayed-type hypersensitivity, but low antibody production; while the functional phenotype of most T_H2 cells may account for the persistent production of antibodies (Romagnani, 1991).

It has been proposed that the balance of T_H1 and T_H2 cells is critical in the development of Type 1 diabetes. This suggestion is made on the basis that at-risk first degree relatives who have poor delayed-type hypersensitivity responses to specific islet antigens (eg glutamic acid decarboxylase, GAD) may be less likely to develop diabetes (Harrison et al., 1993). In addition, there is evidence from the non-obese diabetic (NOD) mouse model of spontaneous diabetes that GAD-responsive, IFN-γ producing T_H1 lymphocytes are an early feature of the disease process (Kaufman et al., 1993).

2.4 CYTOKINES AND AUTOIMMUNE CHRONIC LIVER DISEASE

Autoimmune chronic liver disease (aCLD) is characterised by progressive liver damage associated with autoimmune reactions to specific liver antigens (Vento et al., 1984). The hallmark of aCLD is a mononuclear cell infiltrate present on the portal tract and spilling over into the parenchyma. Activated T lymphocytes and macrophages are conspicuous in this infiltrate (Eggink et al., 1984). It has been suggested that liver damage in aCLD is likely to be orchestrate by $CD4^+$ T lymphocytes which recognise a self antigenic peptide as foreign (reviewed by Mieli-Vergani and Vergani, 1993). During the autoimmune response the peptide is presented to the $CD4^+$ T cells by either a professional antigenic presenting cell such as a macrophage, or by the hepatocyte itself. The T helper cell then becomes activated and can initiate a cascade of immune reactions by producing cytokines,

activating cytotoxic T cells and inducing autoantibody production by B lymphocytes. This model is supported by a number of observations: (a) hepatocytes from patients with active autoimmune hepatitis express class II MHC antigens not normally expressed on liver cells (Lobo-Yeo et al., 1990A) and can therefore present autoantigenic peptides; (b) $CD4^+$ and activated lymphocytes are present in areas of piecemeal necrosis (Colucci et al., 1983; Senaldi et al., 1992); (c) a high proportion of circulating helper T lymphocytes express the activation marker IL-2 receptor (Lobo-Yeo et al., 1990B) and (d) high titre liver-specific autoantibodies are present in the circulation of these patients.

In liver disease, cytokines may contribute to liver cell destruction either directly through their cytotoxic potential or by recruiting inflammatory cells capable of bringing about cell death. Previous studies on hepatitis B virus infected animal model give support to this concept and demonstrated that hepatitis B serface antigen positive transgenic mouse hepatocyte is selectively sensitive to destruction by TNF-α and IFN-γ and this process can be reduced by the prior administration of neutralizing anti-TNF-α and anti-IFN-γ specific monoclonal antibodies (Gilles et al., 1992). A further study also showed that both TNF-α and IFN-γ exerted a cytotoxicity to cultured rat hepatocytes by increasing apoptosis (Shinagawa et al., 1991). These findings indicate that cytokines may play a role in both the orchestration and execution of the immune attack on hepatocytes.

CHAPTER 3

AIMS

3.1 AIM OF THE PRESENT STUDY

The aim of the present study is to investigate the role of cytokines in the development of organ-specific autoimmune disease. Cytokines will be measured in serum and in supernatants of cultured peripheral blood mononuclear cells (PBMC), as well as being detected in situ in the tissues of the target organ. The diseases which will be studied are type 1 diabetes and autoimmune hepatitis. The power of these investigations will be enhanced by access to unique study groups. First, non-diabetic identical twins of patients with type 1 diabetes will be studied prospectively from diagnosis of diabetes in their co-twin. Such non-diabetic twins have a 200-fold higher chance of developing diabetes than the general population, but not all do so. Thus two populations of twins at risk of diabetes will be studied, one of whom becomes diabetic, whilst the other does not, allowing the examination of patterns of cytokine release which presage diabetes. The second population for study is a colony of non-obese diabetic (NOD) mice with a high incidence of spontaneous diabetes.

Specific aims of the project in the investigation of the pathogenesis of type 1 diabetes will be: i) to establish assays to measure the cytokines IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α and sIL-2R at picogram (pg) per millilitre concentration or less; ii) to measure serum levels of IL-1α, IL-1β and TNF-α (macrophage derived cytokines), IL-2 and IFN-γ (T_H1 derived cytokine), IL-4, IL-6 and IL-10 (T_H2 derived cytokine) and sIL-2R in patients with type 1 diabetes of varying disease duration to define if there is a pattern of cytokine alterations characteristic of the disease; iii) to determine whether circulating cytokine levels show a pattern characterising the pre-diabetic period by studying a cohort of non-diabetic identical twins of patients with type 1 diabetes; iv) to examine cytokine levels in first degree relatives of patients with type 1 diabetes, their relationship to HLA type and autoantibodies; v) to investigate cytokine secretion by PBMC from patients with type 1 diabetes stimulated with mitogens in tissue culture; vi) to measure serum levels of TNF-α, IL-2 and IL-4 in NOD mice to define the cytokine profile involved in the development of Type 1 diabetes in this model; vii) to examine cellular expression of cytokines (TNF-α and IFN-γ) in islets of Langerhans of NOD mice of various ages with and without diabetes.

A study of cytokine levels in another organ-specific autoimmune disease, autoimmune hepatitis, will also be performed. i) Circulating levels of cytokines will be measured in children with autoimmune chronic liver disease and chronic liver disease due to metabolic causes, namely Wilson's disease and alpha 1 antitrypsin deficiency as controls; ii) cytokine-producing cells will be examined in the

inflammatory cell infiltrate in liver biopsies from the same children. Cytokine producing cells will be detected in cryostat sections using indirect immunohistochemical or immunofluorescence techniques. The study of cytokines in patients with autoimmune diseases will not only increase our understanding of the role of these factors in disease, but also provide the basis for the utilisation of anti-cytokine therapy (blocking antibodies, soluble receptors) as part of immunotherapy in order to prevent or abrogate autoimmune disease.

CHAPTER 4

MATERIALS AND METHODS

4.1 SUBJECTS WITH DIABETES

In these studies, patients with Type 1 diabetes are defined in accordance with the guidelines of the National Diabetes Data Group (National Diabetes Data Group, 1979), requiring insulin to prevent diabetic ketoacidosis and sustain life. At diagnosis, all had elevated blood glucose and glycosuria. Since collection of blood was usually performed for immunological studies, and bearing in mind that immune function is affected by both metabolic control and intercurrent illness, samples were only obtained from patients who were not in ketoacidosis and had no symptoms or clinical evidence of infection.

4.1.1 PATIENTS WITH TYPE 1 DIABETES

Patients studied were divided into three categories according to the timing of blood sampling in relation to diagnosis of Type 1 diabetes. Patients bled immediately before or within one week of commencement of insulin treatment were considered **newly-diagnosed**. Those sampled within the first year were considered to be within the group of **recent-onset**. Patients with treated Type 1 diabetes for more than 5 years were considered to have **long-standing** disease. Patients were mainly recruited from the Diabetic Department at King's College Hospital, although some were also studied whilst attending clinics at Guy's, Westminster and Farnborough Hospitals. Ethical permission for all studies was obtained from the respective District Health Authority Ethical Committees.

4.1.2 PATIENTS WITH TYPE 2 DIABETES

Unlike type 1 diabetes, patients with type 2 diabetes do not require insulin to prevent ketoacidosis and sustain life. Type 2 diabetics will be studied mainly as "metabolic controls" since, whilst they experience a metabolic imbalance similar to that of Type 1 diabetes, their condition has, almost certainly, an aetiology and pathogenesis distinct from that of type 1 diabetes. All patients studied were attending the Diabetic outpatient clinic at King's College Hospital.

4.1.3 FAMILIES WITH AN INDEX CASE OF TYPE 1 DIABETES

Twenty-nine families, all with an index case of Type 1 diabetes attending the District Diabetes Centre, Farnborough Hospital, volunteered to be bled en masse once in 1989 and once in 1990. As far as possible, samples were obtained from all siblings and both parents: due to the pressures of family life, this was not always possible.

4.1.4 IDENTICAL TWINS OF PATIENTS WITH TYPE 1 DIABETES

Blood samples from monozygotic twins have been collected and stored in the Department of Immunology at King's College School of Medicine and Dentistry for almost 10 years. These samples derive from a unique collection of twin pairs in which at least one of the twins has Type 1 diabetes. The twin collection was begun by Dr David Pyke and has been continued by Dr David Leslie. Zygosity is assessed by typing for the blood groups ABO, CDE, MN, S, P, Lu^a, K, Le^a and Fy^a, which establish monozygosity with a less than 3% chance of error (Cederlöff et al., 1961; Tattersall and Pyke, 1972, Barnett et al., 1981).

The twins studied in this thesis can be allocated into two groups on the basis of the outcome of their risk of developing Type 1 diabetes. In the first group are non-diabetic identical twins of patients with Type 1 diabetes, ascertained within one year of the diagnosis of the index and followed until they themselves became diabetic. This is the **pre-diabetic group**. The second set of subjects are also twins ascertained within one year of the onset of Type 1 diabetes in the index twin, but who had not developed Type 1 diabetes 6 years after this diagnosis. Actuarial analysis has shown that by this time, they have a less than 2% chance of ever developing Type 1 diabetes (Olmos et al., 1988). These form the diabetes-resistant group.

4.2 NON-OBESE DIABETIC MICE

Cytokines were also studied in an animal model of diabetes (non obese diabetic [NOD]) mouse. We have established a colony of NOD mice in the Comparative Biology Unit (CBU) at King's. These animals were obtained from Dr. Hattori, Joslin Diabetes Centre, Boston, U.S.A. The incidence of diabetes in both female (80%) and male (50%) mice is high compared with other centres. During their growth the mice are monitored for weight changes and by weekly urinalysis for glycosuria.

4.3 SUBJECTS WITH AUTOIMMUNE CHRONIC LIVER DISEASE

Children with autoimmune chronic liver disease are considered as another group of patients with organ-specific autoimmune disease. Children were recruited from Child Health outpatient clinic at King's College Hospital. This group includes patients with **autoimmune hepatitis (AIH)** and a form of sclerosing cholangitis, the pathogenesis of which is considered to be autoimmune, **autoimmune sclerosing cholangitis (ASC)** (El-Shabrawi M et al., 1987). They were diagnosed according to

internationally agreed criteria (Scheuer, 1991) and differentiated by the fact that **ASC** had characteristic cholangiographic bile duct changes. Children with other chronic liver diseases such as **Wilson's disease (WD)** and **alpha-1-antitrypsin deficiency (A1ATD)** were studied as pathological controls. Clinical, biochemical, immunological and histological details of the patients with **autoimmune chronic liver disease** are summarised in **table 11.1**.

4.4 HEALTH CONTROLS SUBJECTS

Healthy adults were recruited as control subjects from laboratory, secretarial, medical and portering staff within King's College Hospital and also from blood donors of the South East Thames Regional Blood Transfusion Service. When fresh blood was required for lymphocyte studies, it was drawn from controls at the same time as for the patient group to avoid biases resulting from seasonal, diurnal, or technical variations.

Healthy children were recruited by the Medway and Gillingham Branch (Kent) of the Children's Liver Disease Foundation (CLDF), and gave blood after informed consent from themselves or their parents, as appropriate.

4.5 MATERIALS

4.5.1 REAGENTS

Benzylpenicillin (sodium) BP (Glaxo, Greenford, UK).
Bovine serum albumin (Sigma Chemical Company, Poole, Dorset).
Collagenase type V (Sigma).
3,3-Diaminobenzidine hydrochloride (DAB) (Sigma).
Ficoll-hypaque (Pharmacia, Uppsala, Sweden).
Foetal calf serum, heat inactivated, virus and mycoplasma free (Gibco, Paisley, UK).
Hank's balanced salt solution (Gibco).
Human AB serum, heat inactivated (Donated by Blood Donor whose blood group is AB).
Lauryl sulphate (Sigma).
MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) (Sigma).
O.C.T medium (BDH, Poole, Dorset).
o-phenylenediamine dihydrochloride (OPD)(Sigma).
Preservative-free heparin (Leo Laboratories, Princes Risborough, UK).
Phytoheamoagglutinine (PHA) and Lipopolysaccharride (LPS) (Sigma)
Rabbit, Mouse and Swine serum (Flow Laboratories, Rickmansworth, UK).
RPMI 1640 with glutamine (Gibco).
Streptomycin sulphate (Evans, Greenford, UK)
Tween 20 (Sigma).
All other chemicals were "analar" or at the highest available purity and were obtained from BDH, Sigma, Hopkins and Williams, Chadwell Heath, UK or Fisons, Loughborough, UK

4.5.2 Buffers, solutions and modified media

Blocking buffer (Phosphate Buffered Saline, 1% BSA).
Coating buffer (0.05M Sodium Carbonate pH 9.6).
Chromatography buffers (Chemicals from BDH, Poole, UK):
ion-exchange starting buffer:10 mM Phosphate buffer; pH

8.0; protein-A loading buffer: 1.5 M glycine, 3 M NaCl, pH 8.9; protein-A eluting buffer: 100 mM citric acid, pH 5.0.
Phosphate buffered saline (PBS): 15mM NaCl, 15mM KCl, 8mM Na_2HPO_4,15mM KH_2PO_4, pH 7.2.
RPMI culture medium: RPMI 1640 containing 2mM glutamine, 200 U/ml penicillin, 100 µg/ml streptomycin and 10% foetal calf serum.
Substrate Solution for ELISA: o-phenylenediamine dihydrochloride (OPD). For 20 mls: OPD 8mg, 10mls pyrogen free water, 4.9 mls of 0.01M citric acid, 5.1 mls of 0.2M Na_2HPO_4 (dihydrate) and 300 µl H_2O_2 (6%).
Substrate Solution for Immunohistochemical Staining: 3,3-Diaminobenzidine (DAB). For 5 mls: DAB 3mg and 5mls of PBS. After filtration, 10 µl of H_2O_2 (6%)(w/v) was added (just before using).
Washing buffer (PBS, 0.1% Tween 20, 0.1% BSA).

4.5.3 Consumables

Gelbond plastic film (Marine Colloids, MA, USA).
LP3 tubes (Luckham, Burgess Hill, UK).
Minicon B15 concentrator (Amicon Ltd, Buckinghamshire, UK)
Polystyrene microtitre plates class II and MaxiSorp F96 (Dynatech Immulon, Dynatech Laboratories, Sussex, UK and Nunc Plastic, Poskilde, Denmark).
Tissue culture microtitre plate (Becton Dickinson, Labware, New Jersey, USA).
Tissue culture tubes (Gibco).
"Vacutainers" (plain glass tubes and 'EDTA-tubes') and syringes (Becton Dickinson, Rutheford, NJ, USA).
Visking tubes (Pharmacia).
Universal containers (Sterilin, Teddington, UK).

4.5.4 MONOCLONAL ANTIBODIES (MAb) TO CYTOKINES

Anti-human IL-2 (clone DMS-1) (Genzyme, Genzyme

Diagnostics, Kent, UK).
Anti-human IL-4 (clone 82) (ams Biotechnology Europe Ltd, Oxon, UK).
Anti-human IL-4 labelled with biotin (clone 12) (ams Biotechnology Europe Ltd)
Anti-human IL-6 (clone CLB.IL-6/8) (Lab Impex Diagnostics, Middlesex, UK).
Anti-human IL-6 (clone 1G61) (Dr. Nobuo Ida, Medical Devices and Diagnostics Research Labs, Toray Industries, Kanagawa, Japan).
Anti-human IL-10 (clone JES3-9D7) (ams Biotechnology Europe Ltd).
Anti-human IL-10 labelled with biotin (clone JES3-12G8) (ams Biotechnology Europe Ltd).
Anti-human IFN-γ (clone 69) (Dr. Gallati, Hoffmann-La Roche, Switzerland).
Anti-human IFN-γ peroxidase labelled (clone 123) (Dr. H. Gallati, Hoffmann-La Roche).
Anti-human soluble IL-2 receptor (clone ACT-1, anti-Tac) (Dako, High Wycombe, UK).
Anti-mouse TNF-α (clone TN3 19-12) (Dr. M. Bodmer, Celltech Ltd, UK).
Anti-mouse IL-2 (clone JES6-1A12) (ams Biotechnology Europe Ltd).
Anti-mouse IL-2 labelled with biotin (clone JES6-5H4) ams Biotechnology Europe Ltd).
Anti-mouse IL-4 (clone BVD4-1D11) (ams Biotechnology Europe Ltd).
Anti-mouse IL-4 labelled with biotin (clone BVD6-2492) (ams Biotechnology Europe Ltd).

4.5.5 Polyclonal antibodies to cytokine

Mouse, rabbit and sheep IgG serum (Serotech, UK).
Sheep anti-human IL-1β (Dr. H. Gallati).
Sheep anti-human IL-1β peroxidase labelled (Dr. H. Gallati).

Sheep anti-human IL-1α (S76αBM) (Dr. R. Thorp, National Institute for Biological Standards and Control (NIBSC), Mill Hill, UK).
Rabbit anti-IL-2 (Genzyme).
Rabbit anti-human TNF-α (Dr. M. Bodmer, Celltech Ltd).
Sheep anti-mouse TNF-α (Dr. A. Meager, NIBSC).

4.5.6 Hybridoma cell lines producing antibodies

Mouse hybridoma cell line producing anti-human IL-1α (clone 364/3B3-14) (Dr. R. Thorp, NIBSC).
Mouse hybridoma cell line producing anti-human TNF-α antibody (clone 357-27-8) (Dr. A. Meager, NIBSC).
Mouse hybridoma cell line producing anti-human sIL-2R antibody (7G7/B6) (American Type Culture Collection (ATCC), Rockville, MD, USA).
Rat hybridoma cell line producing anti-mouse IFN-γ antibody (46A4) (Dr. A. Meager).

4.5.7 Cytokines standards

Recombinant human TNF-α (BASF/Knoll, Germany).
Recombinant human IL-1α, IL-1β , IL-2, IL-4, IL-6 and IL-10 (NIBSC).
Recombinant human IFN-γ (Hoffmann-La Roche).
Recombinant mouse TNF-α, IL-2 and IL-4 (NIBSC).
Recombinant mouse IFN-γ (NIBSC).

4.5.8 PEROXIDASE-CONJUGATED ANTISERA

Affinity purified swine anti-rabbit (Dako).
Goat anti-sheep immunoglobulin (Dako).
Rabbit anti-mouse (Dako).
Strept-avidin (Dako).

4.5.9 COMMERCIAL KITS

C-peptide radioimmunoassay (Behringwerke AG, Marburg, Germany).
Particle agglutination kits (Serodia-ATG, Serodia-ACM;

Fujirebio, Tokyo, Japan).

4.5.10 INSTRUMENTS

Cell freezer (Planer products Ltd, Middlesex, UK).
CO_2 Incubator (Leec, Colwick, UK).
-70^{o}C Freezer (Science Temp, Adrian, Mich, USA).
Gamma counter (LKB, 1260 MultiGamma, Bromma, Sweden).
Microelisa reader (MR 700 Microplate Reader, Dynatech).
Mistral 6 L centrifuge (MSE instruments, Crawley, UK).
Mesh (Endecott's, London, UK).
Plate Washer (Dynatech AutoWash 2000, Dynatech).
Polyvar Reichert-Jung fluorescence microscope equipped for epifluorescence (Polyvar, Vienna, Austria).
Peristaltic HiLoad P-50 pump (Pharmacia Biotechnology Ltd, Herts., UK)
Plotter UV-Recorder 2-Channel (Pharmacia Biotechnology Ltd, Herts., UK)

4.6 METHODOLOGY

4.6.1 PREPARATION OF MONOCLONAL ANTI-CYTOKINE ANTIBODIES

Four of the 15 monoclonal antibodies (MAbs) used in the studies of this thesis were prepared "in-house" (monoclonal antibodies to human TNF-α, IL-1α and soluble IL-2 receptor, and to mouse IFN-γ antibodies). They were prepared by culturing antibody producing (anti-cytokine) hybridoma cell lines in vitro, according to the general guidelines reported by Goding (1986). The remaining 11 MAbs were either a gift or purchased. All these were pure and of the IgG class, except for anti-human IL-6 antibodies, which were unpurified MAb, and obtained in ascites fluid.

Purified antibodies were then extracted from both culture supernatants and ascites fluid (i.e. in case of anti-IL-6 MAb) by affinity chromatography (protein-A) and were assessed for the amount and purity of yield, prepared for long-term storage and tested and titrated for subsequent cytokine assays.

Culture of Hybridoma Cell Lines. Hybridomas were thawed on arrival in less than 60 sec in a 37^{o}C water bath and put into culture medium. Immediate culture upon receipt proved to be important in maintaining high cell viability, whilst further storage after arrival reduced viability.

Before initiating culture, the number of viable hybridomas was assessed in a Neubauer's chamber using Trypan blue as an indicator of viability (Hudson and Hay, 1989). Viability ranged in the different lines between 50 and 70% with a median of 60%. Cell numbers were then adjusted in suspension to a concentration of $0.5x10^6$/ml in RPMI 1640 containing 2mM L-glutamine, 200 U/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml amphotericin B and 10% heat-inactivated foetal calf serum (FCS) (from now on referred to as the culture medium) and initially cultured in 25 cm^2 tissue culture flasks in an incubator at 37^{o}C in 5% CO_2. Aliquots of 5 ml of cell suspension were distributed in each flask containing 15 ml culture medium, for further growth and antibody production. Hybridoma cell number and viability as checked daily and 5 ml of fresh culture medium was added to each flask when the number of viable cells exceeded $1x10^6$/ml. When the number of viable hybridoma cells increased to $4x10^6$/ml, they were transferred from 25 into 50 cm^2 tissue culture flasks containing 30 ml of culture medium. Again hybridoma cell number and viability was checked daily so that, by addition of fresh culture medium, now containing 2% FCS, viable cells were maintained in culture at a concentration of $5x10^6$/ml. The decrease of FCS concentration in the culture medium was adopted in order to reduce the final concentration of bovine immunoglobulin, which is only

present in FCS in small amounts, but is sufficient to compromise optimal purity and yield of MAb after chromatography (Manil et al., 1986). The rate of growth varied from cell line to cell line. However, hybridomas reached an adequate number to allow transfer from small (25 cm^2) to bigger flasks (50 cm^2) between approximately 3 and 7 days. This time was found to be the most suitable, since longer periods did not improve antibody yield, decreased hybridoma viability and increased the risk of microbiological contamination, while shorter culture periods reduced the yield of antibodies. The contents of flasks after this culture period were centrifuged at 2000 g for 30 min, the pellet discarded and the supernatant pooled and either purified immediately or stored at -20^oC.

An aliquot of the hybridoma cells obtained after initial growth was removed from culture and suspended in culture medium containing 40% FCS and 10% dimethysulphoxide at a concentration of $5x10^6$/ml. One ml of this cell suspension was immediately frozen in a controlled rate cell freezer (-1 ^oC/ minute) and stored in liquid nitrogen (-70^oC) for future needs.

Purification of Monoclonal and Polyclonal anti-cytokine Antibodies. MAb obtained in ascitic fluid or supernatant after hybridoma culture in vitro was purified by affinity (protein-A) chromatography. Ion-exchange chromatography was used to purify polyclonal antibodies. Chromatography was always performed in columns at room temperature.

Protein-A chromatography. Protein-A is a protein from Staphylococcus aureus which binds to the Fc portion of IgG from a wide range of species (Goudswaard et al., 1978). Protein-A is covalently coupled to a cross-linked matrix, Sepharose CL-4B. This matrix exhibits high chemical and mechanical stability (Ey et al., 1978).

The IgG binding properties of protein A make chromatography with protein A-Sepharose CL-4B immunoadsorbents a very simple method for preparing IgG since it has excellent purification properties in terms of both the amount and purity of yield, whilst at the same time being rapid to perform (Manil et al., 1986).

Protein A-Sepharose CL-4B beads were hydrated with saline solution, poured, packed and equilibrated with loading buffer in a chromatography column according to manufacturer's instructions. A 5 cm length x 1 cm diameter column was prepared, which is able to bind 25 mg of murine immunoglobulin.

After recovery from culture or following storage at -20^oC, 200 ml of supernatant was filtered using 0.22 μm micropore filters and mixed with an equal volume of loading buffer for affinity chromatography with protein-A. The

composition and pH of the loading (alkaline) and eluting (acid) buffers were those recommended by Pharmacia (Mariani *et al*., 1989). Adjusted to the optimal pH of 8.9, the mixture of supernatant and buffer was loaded on the column at a flow rate of 30 ml/hour controlled by a peristaltic pump. The eluent was monitored for protein content with an UV (280 nm absorbance) spectrophotometer connected to a plotter (**Figure 4.1, phase 1**). Once the mixture of supernatant and buffer had passed through the column, it was rinsed with approximately 200 ml of loading buffer alone flowing at the same speed. During this procedure no protein eluted from the column (**Figure 4.1, phase 2**). At this stage the column was treated with the eluting buffer at pH 3.0, flowing at the same rate as above, to yield the MAb retained by protein-A (**Figure 4.1, phase 3**). 20 ml of eluting buffer was sufficient to remove the MAb from the protein-A column completely; the eluent was collected in fractions of 5 ml. Each fraction neutralised with 5 ml of 1 M carbonate buffer at pH 8.0, to prevent degradation occurring in the acid conditions. The fractions were then pooled and concentrated by ultrafiltration to a final volume of 5 ml which were dialysed overnight at 4^{o}C against 1 litre of PBS containing 0.01% sodium azide and changed twice. MAb present in this final solution was assessed for yield, tested and titrated for cytokine assays and stored as described below.

Purification of MAb obtained in ascitic fluid, as in case of MAb anti-human IL-6 antibodies, was achieved in similar manner to purification of MAb obtained in supernatants of tissue culture using protein A chromatography.

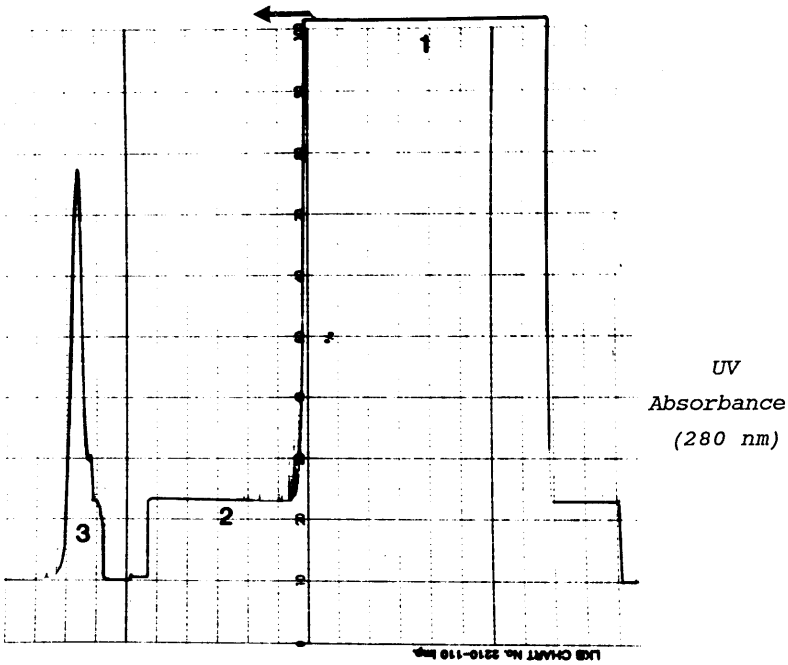


Figure 4.1. Curve describing the protein content in the fluid leaving the protein A chromatography column as determined with an UV spectrophotometer (280 nm absorbance). On the vertical axis is the absorbance and on the horizontal axis the time. When initially the mixture of supernatant and buffer was loaded on the column a high content of proteins, deriving from foetal calf serum in the supernatant, was found (phase 1). When subsequently loading buffer alone was loaded the protein content gradually declined (phase 2). When the eluting buffer was loaded on the column the monoclonal antibody retained by protein-A was released and found in the eluate (phase 3).

Ion-exchange chromatography. This method was preferred to protein-A chromatography for the purification of the IgG class polyclonal antibodies (raised in sheep: anti-IL-1 and anti-IL-6 or in rabbit: anti-TNF). In this method, proteins are bound electrostatically on to an ion-exchange matrix bearing an opposite charge. The degree to which a protein binds depends upon its charge density. Proteins are then eluted differentially by:

(a) Increasing the ionic strength of the buffer running through the column. As the concentration of buffer ions is increased proteins are displaced from the charged groups on the ion-exchanger.

(b) Alteration of the pH. As the pH of the buffer approaches the isoelectric point of each protein, the net charge on the protein becomes zero and so the protein no longer

binds to the ion-exchanger.

This technique offers the potential for separation of one immunoglobulin class from others, as is the case of a polyclonal antibodies in hyperimmunised animals (Hudson and Hay, 1989).

A matrix for anionic ion-exchange chromatography (DEAE-Sephacel) was obtained pre-swollen. A column of 40 cm length and 1.5 cm diameter was packed according to the manufacturer's instructions and equilibrated with starting buffer. The size of the column was selected on the basis of the volume and viscosity of the sample to be purified. The composition and pH of the starting buffers were used according to the technique by Hudson and Hay (1989).

Polyclonal serum was prepared for chromatography by overnight dialysis at $4^{o}C$ of 5 ml serum against 1 l of starting buffer. The sample was then loaded and the column flushed through with three column volumes of starting buffer. IgG was eluted in a single asymmetric peak (**Figure 4.2**) and impurities were bound and retarded by the exchanger. The eluate was collected and the fractions comprising the first protein peak were pooled and dialysed as for the fractions obtained with protein-A chromatography and finally diluted in PBS with 0.01% sodium azide to a final volume of 5 ml. The MAb and polyclonal antibodies obtained after purification were further processed as described below.

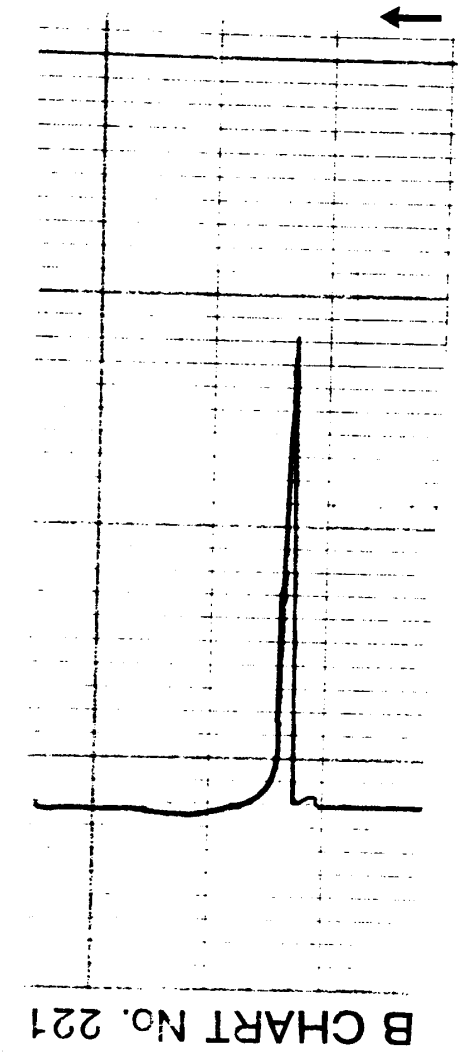


Figure 4.2. Isolation of IgG fraction from sheep anti-IL-1α serum on DEAE-Sephacel chromatogram. On the vertical axis is the absorbance (280 nm) and on the horizontal axis is the time. The peak shown was obtained by elution of IgG fraction with 10 mM phosphate buffer, pH 8.0.

Assessment of amount and purity of monoclonal and poly-clonal antibodies. This procedure was necessary to ascertain the performance, in both quantitative and qualitative terms, of the MAb and polyclonal production and purification, and to allow appropriate dilution for storage and titration.

Protein content of the final MAb and polyclonal solutions recovered after protein-A or ion-exchange chromatography was measured by a Coomassie blue method (Bradford, 1976) using a commercially available kit and following

manufacturers's instructions. It was calculated that each ml of culture supernatant contained between 100-300 μg of MAb (median 230 μg), while each ml of polyclonal serum contained 7-10 mg (median 8 mg).

To evaluate the efficiency of extraction of MAb, mouse immunoglobulin content was measured in both the original solution (supernatant, ascites) and the purified final product by radial immunodiffusion. A gel containing 1% agarose, 2% polyethylene glycol and 1% rabbit anti-mouse immunoglobulin was cast on to a plastic support medium (Gelbond) in a template to give a final thickness of 1.5 mm. Thirty wells of 2 mm diameter were punched and loaded with 10 μl from (A) neat supernatants, (B) ascites diluted 1/400 in PBS, (C) purified protein solutions (A and B) diluted 1/40 in PBS and (D) six doubling dilutions of standard mouse serum in PBS from 1/100 to 1/3200 to obtain a reference curve. After a 48 hour incubation at 4^{o}C, diameters of the precipitation rings formed around each well were measured, the reference curve plotted and the unknown concentrations derived. The results were expressed as percentages of the maximum standard (100%). A similar approach was adopted to evaluate the efficiency of extraction of polyclonal antibodies of sheep and rabbit immunoglobulins. Pre:post-purification ratios were calculated, to yield the percentages of extracted MAb and polyclonal antibody. These ranged between 43% and 93% and had a median of 81%.

Storage of monoclonal and polyclonal antibodies. All antibodies were adjusted to final concentration of approximately 1 mg/ml using PBS containing 0.01% sodium azide. They were then divided into 1 ml aliquots and stored at -70^{o}C.

Biotin-conjugation. This technique was required to conjugate MAb anti-IL-6 to biotin which was used in the revealing step of IL-6 immunoassay as described later (vide 4.6.2). The conjugation was performed according to the technique of Bayer and Wilchek (1980) and at relatively low protein concentration (1 mg/ml).

Biotin hydroxy succinimide ester (BHSE) was dissolved immediately before use in dimethyl sulfoxide (DMSO) at a final concentration of 50 mg/ml. Purified anti-IL-6 MAb was adjusted to 1 mg/ml and dialyzed in "Visking" tubes against 1 litre of 0.1M $NaHCO_3$ buffered, pH 9.5. BHSE solution was added dropwise to the anti-IL-6 preparation at a protein/BHSE ratio of 1mg/ 1mg. The mixture was kept agitated for 15 minutes, and then allowed to stand for 180 minutes at room temperature. The unbound biotin was removed by dialysis against 20 ml of PBS using a Minicon B15 concentrator. Biotinylated anti-IL-6 MAb was then aliquoted and stored at -70^{o}C. Optimum conjugate concentrations of anti-IL-6 MAb used in the immunoassay were determined as described for standardisation methodology for

the indirect ELISA of TNF-α (vide 4.6.1.6).

Specificity of Anti-cytokine Antibodies. A direct enzyme linked immuno-sorbent assay (ELISA) was used to check whether a given antibody reacts specifically with a corresponding cytokine. This technique was performed according to Lamche and Adolf (1990). Microtitre plates were coated (16 hour, 4°C) with corresponding recombinant cytokine (30 ng/100 µl carbonate buffered solution, pH 9.5, per well). After washing the plates (0.05% Tween 20 in PBS) and treating them with 1% bovine serum albumin (BSA) (60 min, at room temperature to block non-specific binding sites), 100 µl of 1/10 dilution of each antibody (monoclonal and polyclonal anti-cytokine antibodies) was added to the wells of corresponding and irrelevant cytokines. After 60 min at 37°C, the plates were washed and incubated (60 min at 37°C) with horseradish peroxidase-conjugated anti-species sera (e.g. goat anti-mouse, goat anti-rabbit or rabbit anti-sheep), followed by addition of the enzyme substrate, o-phenylenediamine dihydrochloride (OPD) (vide 4.5). Optical density (OD) at 490 was determined after a 15-30 min incubation at room temperature using Microelisa Auto Reader. All anti-cytokines antibodies reacted specifically with corresponding cytokine antigens and did not cross react with irrelevant cytokine (**Table 4.1)**.

Table 4.1 Means of Absorbance reading at 490 nm for anti-cytokine MAb (1/10) reactions when used in plate coated with 30 ng/ml of different cytokines (e.g. human TNF-α, human IL-1α, human sIL-2R or mouse IFN-γ) in the direct ELISA technique.

1/10 dilution of anti-cytokine antibody	**Human TNF-α**	**Human IL-1α**	**Mouse IFN-γ**	**Human sIL-2R**
Anti-human TNF-α MAb	0.781	0.009	0.002	0.000
Anti-human IL-1α MAb	0.001	0.562	0.001	0.001
Anti-mouse IFN-γ MAb	0.000	0.005	0.925	0.000
Anti-human sIL-2R MAb	0.001	0.001	0.000	0.458

Test and Titration of Monoclonal and Polyclonal Antibodies used in cytokine assays. These procedures have aim to establish that the functional capability of the antibody has been preserved during culture and purification and to find the optimal concentrations for use in cytokine assays comprising immunoassay and

immunohistochemical staining. These techniques were as described for standardisation methodology of TNF-α immunoassay (vide 4.6.2) and immunohistochemical staining of TNF-α in the liver (vide 4.6.5).

4.6.2 CYTOKINE ASSAYS

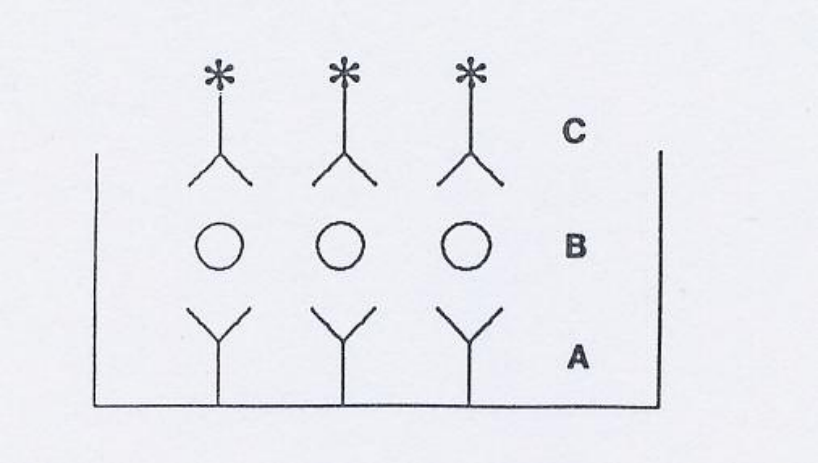
Levels of cytokines were measured in the serum or in the supernatant of stimulated peripheral blood mononuclear cells (PBMC) using either ELISA or bioassay, while cytokine producing cells were detected in cryostat tissue sections using indirect immunohistochemical or immunofluorescence technique.

4.6.2.1 CYTOKINE IMMUNOASSAY

An ELISA technique for measurement of cytokine levels in biological fluid including serum has been introduced by Meager et al. (1987). This technique offers a number of advantages compared with bioassay and radioimmunoassay (Meager et al., 1987): it can be performed in short time and without using radiolabelled materials, multiwell microtitre plates used as the solid phase are easy to handle and wash, and when used with automated readers and multiple well washers allow a large number of samples to be assayed (reviewed by Kemeny and Chantler, 1988).

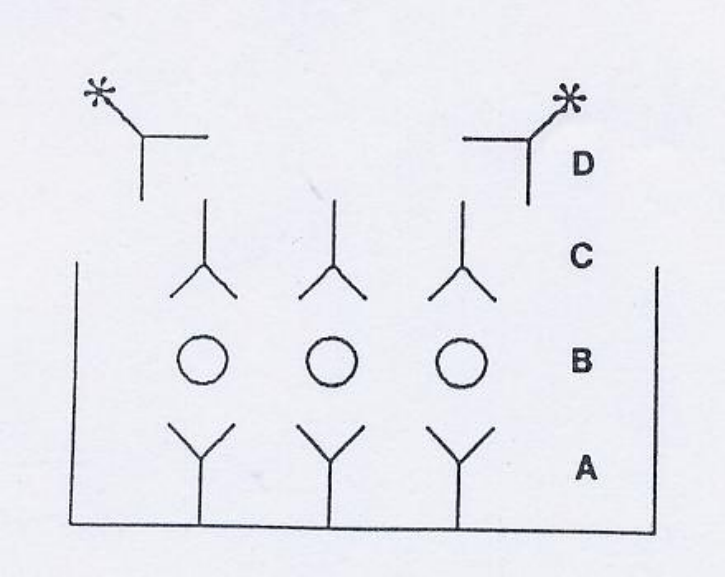
For the measurement of cytokines, a two-site ELISA was used where the antibody (i.e. anti-cytokine antibody) was employed on the solid phase to capture the corresponding antigen (cytokine) in the test sample. The concentration of cytokine in a sample was determined by the amount of enzyme-labelled anti-cytokine IgG bound to the cytokine captured by the first, immobilized, anti-cytokine antibody **(Figure 4.3A)**. If the revealing antibody was unlabelled, a subsequent addition of enzyme-labelled anti-species antibody was used **(Figure 4.3B).**

Monoclonal anti-cytokine antibodies to different group-common epitopes on the analyte (cytokine) can be used or, alternatively, combinations of monoclonal and polyclonal reagents may be used for capture and detector phases of the cytokine immunoassay (Clayton et al., 1986; Kemeny and Richards, 1987). All labelled antibodies employed in the revealing steps of the cytokine immunoassays were HRP-labelled antibodies, and O-orthophenylene diamine (OPD) was used as a substrate for the of HRP enzyme.



4A

A = "capture anti-cytokine antibody" adsorbed onto a microtitre well.
B = cytokine (standards or test samples).
C = enzyme labelled anti-cytokine antibody.



4B

A = "capture anti-cytokine antibody" adsorbed onto microtitre well.
B = cytokine (standards or test samples)
C = unlabelled revealing anti-cytokine antibody .
D = enzyme labelled anti-species antibody.

Figures 4.3 (A and B). A two-site ELISA for measurement of cytokines.

Assay optimization for TNF-α and IL-1α immunoassays.

The optimum concentration of the various components is determined by chequerboard titration, initially using a wide range of dilutions to identify the working range of the assay and subsequently small dilution increments around the optimum to determine the precise concentration (Kemeny and Chantler, 1988).

- **Determination of Optimum anti-TNF-α and IL-1α monoclonal antibodies concentrations for coating of microtitre plates.**

100 µl of monoclonal anti-TNF-α or anti-IL-1α antibodies diluted in coating buffer (0.1M bicarbonate buffer (pH 9.5)) at various concentrations (0.5, 1, 2.5 and 5 µg/well) were coated onto ELISA plates overnight at 4°C. Plates were washed 3 times in phosphate buffered saline (PBS) supplemented with 0.1% bovine serum albumin and 0.01% Tween 20 (washing buffer) and non-specific binding sites were blocked by incubation with blocking buffer (PBS with 1% BSA) for 1 hour at room temperature. After washing, 100 µl of standard (recombinant TNF-α and IL-1α) was added for 2 hour at room temperature with constant shaking. Plates were then washed and 100 µl polyclonal rabbit anti-TNF-α or sheep anti-IL-1α antibodies at concentrations of 1 µg/ml and 0.5 µg/ml respectively diluted in the dilution buffer and supplemented with 2% mouse serum (Meager et al., 1987) (to block possible non-specific reaction) were added and incubated for 2 hour at room temperature with constant shaking. After washing, 100 µl of horseradish peroxidase-conjugated goat anti-rabbit or anti-sheep immunoglobulins were added in a dilution of 1/2000, supplemented with 2% mouse serum and incubated for 1 hour at room temperature with constant shaking. The reaction was developed using 100 µl of substrate (0.4 mg/ml o-phenylenediamine in citrate phosphate buffer (pH 5.0)) containing 4 µl/ml of 3% hydrogen peroxide for 30 minutes in the dark at room temperature. The reaction was stopped with 50 µl of 4N H_2SO_4 and the absorbance at 490 nm was read in a Dynatech plate-reader. Tests were performed in triplicate and unknown values read from standard curves constructed using recombinant TNF-α and IL-1α at a range of dilutions from 7.5-1920 pg/ml.

The results obtained indicated that the maximum response (OD) was given by 2.5 µg/ml for both TNF-α and IL-1α. No increase was found at highest concentration, hence 2.5 µg/ml was determined to be the optimum concentration (**Figure 4.4**).

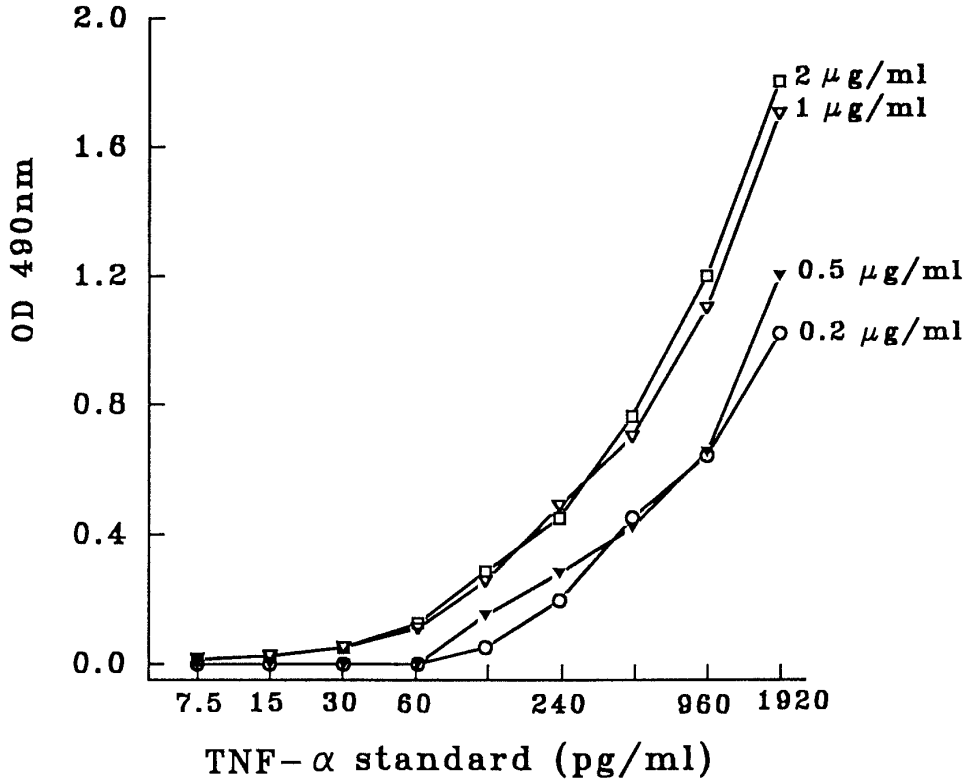


Figure 4.4. TNF-α standard curves using a range of concentrations (0.5, 1, 2.5 and 5 μg/well) of anti-TNF-α MAb in the capture step in the TNF immunoassay. Polyclonal rabbit anti-TNF-α and horseradish peroxidase-conjugated anti-rabbit antibodies were used in the revealing step at a concentration of 1 μg/ml. Standard curves were constructed using recombinant TNF-α at a range of serial doubling dilutions from 7.5-1920 pg/ml (horizontal axis) and optical density at 490 nm was read in an ELISA reader (vertical axis). Optimal concentration of coating antibody was found to be 2.5 μg/ml.

● **Determination of optimum incubation times for cytokine/anti-cytokine MAb (Ab1) reaction of TNF-α and IL-1α immunoassays.**

A range of incubation times (1, 2 and 18 hours) for the cytokine/anti-cytokine MAb reaction were investigated for both TNF-α and IL-1α immunoassays. From the results shown in **Table 4.2**, optimum binding was found after 2 hour incubation for both TNF-α and IL-1α.

Table 4.2 The mean absorbance at 490 nm for TNF-α standards at a range of dilutions from

7.5-1920 pg/ml after different TNF-α/anti-TNF-α MAb (Ab1) reaction incubation times.

Incubation time	Concentration of TNF-α (pg/ml)				
	7.5	**30**	**120**	**480**	**1920**
1 hour	0.000	0.065	0.215	0.758	1.542
2 hour	0.015	0.092	0.329	1.070	2.456
overnight (18 hour)	0.009	0.086	0.289	1.056	1.952

- **Determination of optimum anti-TNF-α and IL-1α polyclonal antibodies concentrations used in the revealing step of the TNF-α and IL-1α immunoassays.**

Having optimised anti-TNF-α and IL-1α MAb concentrations employed in the coating step, a range of concentrations of anti-TNF-α and IL-1α polyclonal antibodies (0.2, 0.5, 1 and 2 μg/ml) were then tested in the revealing step of the immunoassay as described above. The results obtained indicated that the appropriate polyclonal antibodies concentrations used in the revealing steps were found to be 1 μg/ml for TNF-α (**Figure 4.5**) and 0.5 μg/ml for IL-1α which provided high absorbance values for TNF-α and IL-1α standards and constant low values for nonspecific background.

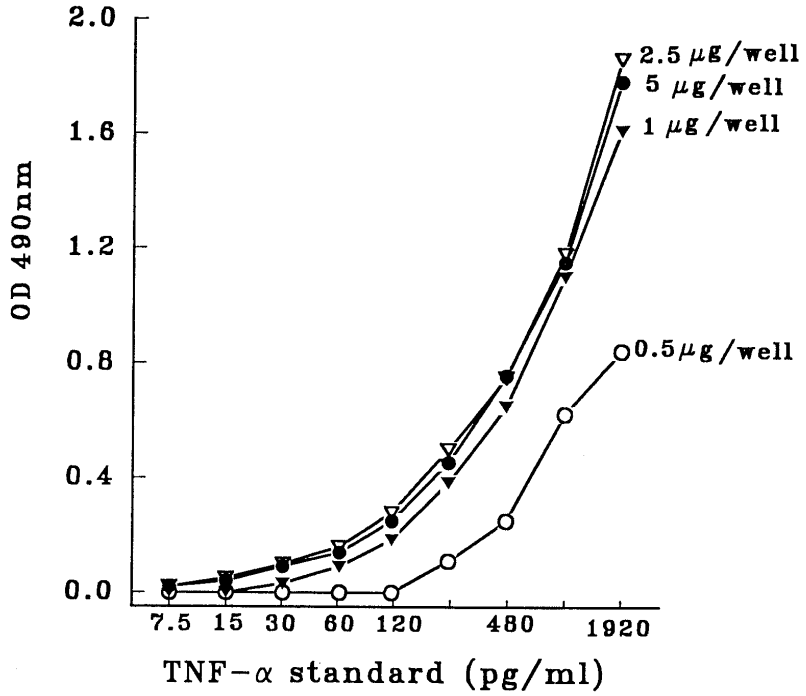


Figure 4.5. TNF-α standard curves using a range of concentrations (0.2, 0.5, 1 and 2 μg/ml) of anti-TNF-α polyclonal antibodies in the revealing step of the TNF-α immunoassay. The plate was coated with capture MAb anti-TNFα at a concentration of 2.5 μg/well. Standard curves were constructed using recombinant TNF-α at a range of serial doubling dilutions from 7.5-1920 pg/ml (horizontal axis) and optical density at 490 nm was read in an ELISA

reader (vertical axis). The optimal concentration of revealing antibody was found to be 1 μg/ml.

● **Determination of optimum conjugate concentration.**

Horseradish peroxidase-conjugated goat anti-rabbit or anti-sheep immunoglobulins used in the immunoassay were diluted 1/2000 in the dilution buffer as recommended by the manufacturers. The results shown in **Figure 4.4-4.6**, were found most satisfactory with maximal binding of cytokines and with negligible background for the blank wells.

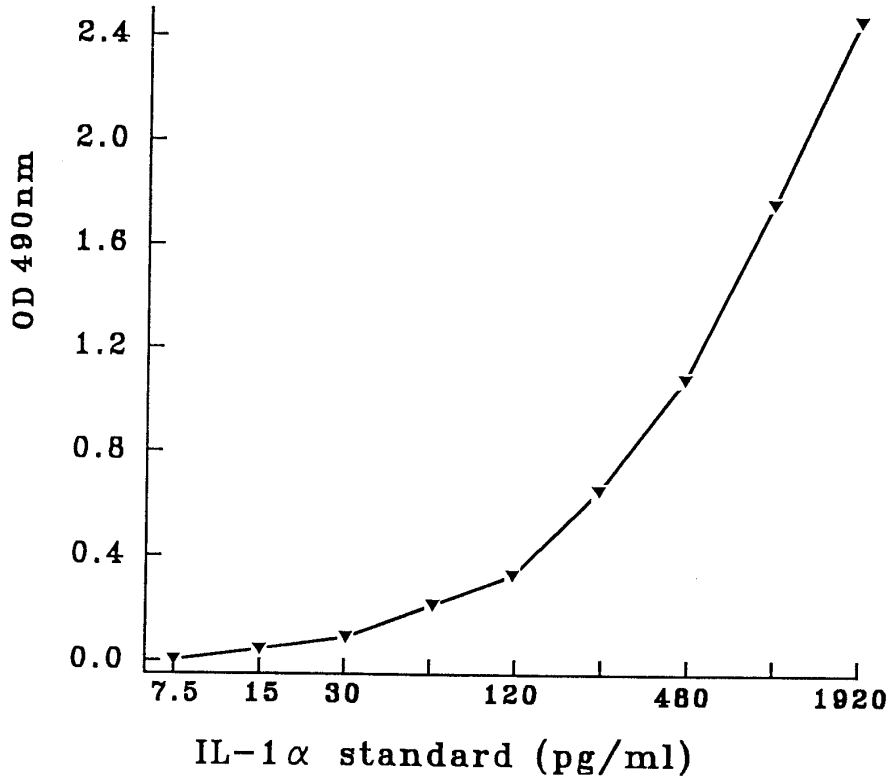


Figure 4.6. A standard curve of IL-1α immunoassay. The plate was coated with anti-IL-1α monoclonal antibody at a concentration of 2.5 μg/well. A polyclonal sheep anti-IL-1α and polyclonal horseradish peroxidase-conjugated anti-sheep antibodies were used in the revealing step at a concentration of 0.5 μg/ml and 1 μg/ml respectively. The plot represents the absorbances at 490 nm (vertical axis) versus the standard of IL-1α at a range of dilutions from 7.5-1920 pg/ml (horizontal axis).

Table 4.3 and 4.4 show optimum concentrations of the monoclonal and polyclonal antibodies used in the coating and revealing steps for cytokines studied such as IL-1β, IL-6, IFN-γ and sIL-2R as determined in similar manner to those of the TNF-α and IL-1α immunoassays, the optimal concentration were adopted for use in cytokine immunoassays.

Optimum dilutions of anti-cytokine and conjugated anti-species antibodies used for detection of cytokine producing cells were determined using a chequerboard titration test (Kemeny and Chantler, 1988) in similar manner to that for immunoassays using the two-step indirect immunohistochemical or immunofluorescence staining techniques (vide 4.6.4)

(**Table 4.5**). The specificity of the staining was determined by prior incubation of anti-cytokine antibody with 100 μg/ml of corresponding cytokine, which resulted in abolition of peroxidase staining.

Table 4.3 Monoclonal and polyclonal anti-human cytokine antibodies purchased or donated and used in immunoassay.

Antibody	**Form**	**Concentrat-ion***	**Source**	**purpose of use**
Monoclonal anti-IFN-γ	Ascitic fluid	5 μg/well	Hoffmann-La Roche	Coating step
Monoclonal anti-IFN-γ HRP labelled	Ascitic fluid	1 μg/ml	Hoffmann-La Roche	Revealing step
Polyclonal anti-IL-1β	Sheep serum	5 μg/well	Hoffmann-La Roche	Coating step
Polyclonal anti-IL-1β HRP labelled	Sheep serum	1 μg/ml	Hoffmann-La Roche	Revealing step
Monoclonal anti-sIL-2R biotin labelled	Ascitic fluid	2 μg/ml	Dako	Revealing step
Polyclonal anti-IL-1α	Sheep serum	0.5 μg/ml	NIBSC	Revealing step
Polyclonal anti-TNF-α	Rabbit serum	1 μg/ml	Celltech	Revealing step
Monoclonal anti-IL-6	Ascitic fluid	1.5 μg/well	Lab Impex Diagnostics	Coating step
Monoclonal anti-IL-6 biotin labelled	Ascitic fluid	2 μg/ml	Toray Industries	Revealing step
Monoclonal anti-IL-4	Ascitic fluid	1 μg/well	ams Biotechno-logy	Coating step
Monoclonal anti-IL-4 biotin labelled	Ascitic fluid	1 μg/ml	ams Biotechno-logy	Revealing step
Monoclonal anti-IL-10	Ascitic fluid	1 μg/well	ams Biotechno-logy	Coating step
Monoclonal anti-IL-10 biotin labelled	Ascitic fluid	1 μg/ml	ams Biotechno-logy	Revealing step

***** Concentrations of coating antibodies given as μg/well while concentration of revealing antibodies given as μg/ml.

Table 4.4 Monoclonal anti-human cytokine antibodies prepared and used in cytokine immunoassay.

Monoclonal Antibody	Form	Clone	Concentration*	Purpose of use
Anti-TNF-α	Culture supernatant	357-27-8	2.5 μg/well	Coating layer
Anti-sIL-2R	Culture supernatant	7G7B6	5 μg/well	Coating layer
Anti-IL-1α	Culture supernatant	3B3-14	2.5 μg/well	Coating layer

***** Concentrations of coating antibodies given as μg/well while concentration of revealing antibodies given as μg/ml.

Table 4.5 Monoclonal and polyclonal anti-cytokine antibodies prepared or donated and the optimum dilution used in immunohistochemical staining.

Antibody	Form	Titre*	Source
Monoclonal anti-human TNF-α (clone 27)	Culture supernatant (prepared)	1/10	NIBSC
Monoclonal anti-human IFN-γ	Ascitic fluid (donated)	1/20	Hoffmann- La Roche
Polyclonal anti-mouse TNF-α	Sheep serum (donated)	1/200	NIBSC
Monoclonal anti-mouse IFN-γ	Culture supernatant (prepared)	1/10	NIBSC

***** Antibody titre of the original protein concentration of 1 mg/ml

Intra-assay and inter-assay variations. The cytokine immunoassay was tested for the intra-assay and inter-assay coefficients of variation (CV) using seven serum samples. These were tested in triplicate for intra-assay variation and eight runs for inter-assay variation. An example for the intra-assay and inter-assay variation of TNF-α is shown in **Table 4.6 and 4.7** Mean CV values were ranged between 3.7%-9.1% for intra-assay and 8%-15% for inter-assay.

Table 4.6 Intra-assay variation for TNF-α of 7 serum samples which were tested in triplicate.

Sample no.	**Mean (pg/ml)**	**Standard Deviation (pg/ml)**	**Coefficient of Variation (%)**
1	28	3	9
2	35	3	9.1
3	40	2	5
4	142	7	5
5	237	10	4.2
6	515	18	4
7	620	23	3.7

Table 4.7 Inter-assay variation for TNF-α of 7 serum samples which were tested for eight runs.

Sample no.	Mean (pg/ml)	Standard Deviation (pg/ml)	Coefficient of Variation (%)
1	12	1.8	15
2	35	4	11.4
3	49	5	10.2
4	75	7	9.3
5	120	14	9.1
6	162	13	8
7	354	29	8.2

Recovery of exogenous TNF-α and IL-1α from serum samples. In order to examine whether this ELISA is suitable for use with biological fluids, the following experiments were performed. Human recombinant TNF-α or IL-1α was added to serum samples obtained from 7 apparently healthy subjects, at a final concentration of 500 pg/ml and assayed in ELISA. **Table 4.8** shows the results of a recovery test for IL-1α from serum samples. The mean percentage recovery of TNF-α and IL-1α from 7 different serum samples was >90%, indicating that the inhibition by serum components was negligible.

An example for the percentage recovery of TNF-α is shown in **Table 4.8.**

%Recovery was calculated as:

[{Observed TNF-baseline TNF}- Amount of TNF added]X 100%

Table 4.8 The percentage recovery of exogenous TNF-α from serum sample.

Sample no.	**Without TNF-α addition**	**500 pg/ml TNF-α added**	**TNF-α recovered (pg/ml)**	**% recovered**
1	0	500	485	97.0
2	0	500	432	86.4
3	0	500	493	98.6
4	0	500	429	85.8
5	0	500	468	93.6
6	0	500	480	96.0
7	0	500	434	86.8

Sensitivity of TNF-α and IL-1α immunoassays. The sensitivity of the immunoassay for TNF-α and IL-1α was calculated and found to be 7.5 pg/ml (**Table 4.9**). The plot of the absorbance versus IL-1α concentration in picograms/ml (pg/ml) for the standards is shown in **Figure 4.6**. When OD values were below the lowest value of the reference curve, a value of 0 pg/ml was assigned.

Table 4.9 The absorbance at 490 nm for TNF-α standards at a range of dilutions from 7.5-2000 pg/ml.

TNF-α standard	Concentration (pg/ml)	OD 490 nm (triplicate)	Average OD 490 nm
Standard 1A,1B	0 (Blank)	0.001, 0.001, 0.003	0.002
Standard 2A,2B	7.5	0.012, 0.014, 0.018	0.015
Standard 3A,3B	15	0.042, 0.036, 0.042	0.040
Standard 4A,4B	30	0.090, 0.092, 0.094	0.092
Standard 5A,5B	60	0.217, 0.220, 0.236	0.224
Standard 6A,6B	120	0.335, 0.323, 0.319	0.325
Standard 7A,7B	240	0.652, 0.652, 0.650	0.651
Standard 8A,8B	480	1.060, 1.080, 1.075	1.070
Standard 9A,9B	960	1.762, 1.754, 1.724	1.746
Standard 10A,10B	2000	2.450, 2.412, 2.462	2.441

Measurements of IFN-γ and IL-1β. Interferon-γ and IL-1β were measured according to the technique of two-site ELISA by Gallati et al. (1988) using combinations of anti-IL-1β coating antibody and peroxidase labelled anti-IL-1β polyclonal revealing antibodies for IL-1β immunoassay and anti-IFN-γ coating

antibody and peroxidase labelled anti-IFN-γ monoclonal revealing antibodies for IFN-γ. A one-step incubation was adopted in this assay since the detecting antibody used in the revealing step was conjugated with a peroxidase (i.e. detecting antibody was added immediately after the cytokine containing sample). The optimum concentrations of these antibodies and incubation time used in the immunoassay were determined in similar way to TNF-α immunoassay.

Microtitre plates were coated with 100 µl murine anti-IFN-γ monoclonal antibody and goat anti-IL-1β antibody diluted in 0.1M bicarbonate buffer (pH 9.5) to a concentration of 5 µg/well for both. Plates were then incubated at 4^{o}C for 24 h, followed by incubation at 37^{o}C for 1 hour with 100 µl blocking buffer to eliminate non-specific binding to the plastic wells. Serial dilutions of standards or test samples in dilution buffer were added (final volume 100 µl/well). 50 µl of horseradish peroxidase-conjugated monoclonal anti-IFN-γ and polyclonal anti-IL-1β immunoglobulins diluted in PBS buffered containing 0.1% BSA to a concentration of 1 µg/ml were added immediately after the cytokine containing sample and incubated overnight at 4^{o}C. After washing, the reaction was developed as described for TNF-α and IL-1α immunoassays. Tests were performed in duplicate and unknown values read from standard curves constructed using recombinant IFN-γ and IL-1β. The standard curve ranged between 15-1920 pg/ml (**Figure 4.7**). The sensitivity of the assay was 30 pg/ml for both IFN-γ and IL-1β. No cross-reaction for either IFN-γ and IL-1β assays with other cytokines such as IL-1α, TNF-α, IL-4 and IL-10 was found as determined methodology similar to that for the TNF-α immunoassay. The intra-assay and inter-assay variations (%) for both IL-1β and IFN-γ ranged between 8%-15%

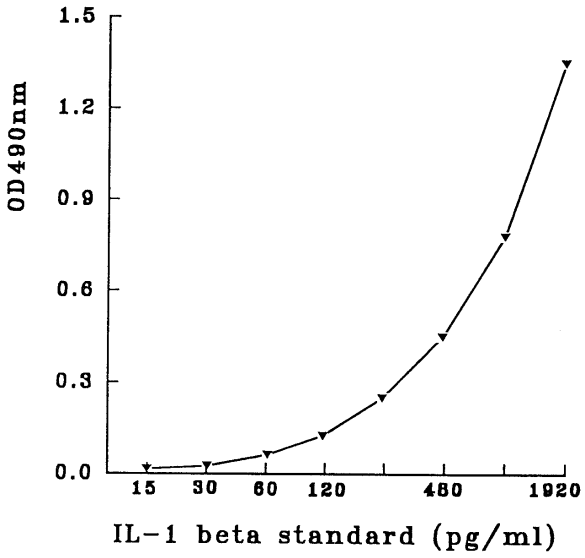


Figure 4.7. A standard curve of IL-1β immunoassay. The plate was coated with anti-IL-1β monoclonal antibody at a concentration of 1 μg/well and horseradish peroxidase- conjugated anti-IL-1β polyclonal antibody was added immediately after the cytokine containing sample and incubated overnight at 4°C. The plot represents the absorbances at 490 nm (vertical axis) versus the standard of IL-1β at a range of dilutions from 15-1920 pg/ml (horizontal axis).

Measurement of IL-6 and soluble IL-2 receptor (sIL-2R). The determination of IL-6 and soluble IL-2R levels was performed by the technique of two-site ELISA by Aarden et al. (1992) and Rubin et al. (1985a) in which combinations of two monoclonal antibodies (i.e. clones 8 and 1G61 for IL-6 and clones 7G7B6 and anti-TAC for sIL-2R) were used in the coating and revealing steps respectively each clone recognises different epitope of either IL-2R or IL-6 respectively. The second antibodies used in the revealing step (i.e. anti-sIL-2R (anti-TAC) and anti-IL-6 (1G61)) were labelled with biotin and the reaction was developed by addition of peroxidase-conjugated streptavidin. The optimum concentrations of coating and revealing antibodies were determined as described for TNF-α immunoassays.

Wells of flat-bottomed 96-well microtitre plates were coated with 100 μl purified anti-sIL-2R (7G7B6) and anti-IL-6 (clone CLB. IL-6/8) antibodies overnight in coating buffer at optimal concentration of 5 μg/well and 1.5 μg/well

respectively. After washing, 100 µl of test samples or standard were added to duplicated antibody-coated wells. After a 2 hour incubation at 37°C, the plates were washed with washing buffer and 100 µl of second biotinylated monoclonal anti-sIL-2R (anti-TAC) and anti-IL-6 (1G61) antibodies diluted in the dilution buffer and supplemented with 2% mouse serum to a final concentration of 2 µg/ml (to block possible non-specific reaction), were added into each well. After an additional 2 hour incubation at room temperature, plates were washed and 100 µl of horseradish peroxidase-conjugated streptavidin was added at a dilution of 1/2000 and supplemented with 2% mouse serum. The wells were then incubated for 1 hour at room temperature with constant shaking. The reaction was developed after washing as described for TNF-α and IL-1α immunoassays (vide 4.6.2). Tests were performed in duplicate and unknown values read from standard curves constructed using recombinant IL-2R and IL-6 (**Figures 4.8 and 4.9**). The sensitivity of the assay was 30 U/ml for sIL-2R and 7.5 pg/ml for IL-6. No cross reaction was observed with other cytokines such as IL-1α, TNF-α and IFN-γ. Intra-assay and inter-assay coefficient of variation (%) of test samples ranged between 7%-13% for both sIL-2R and IL-6.

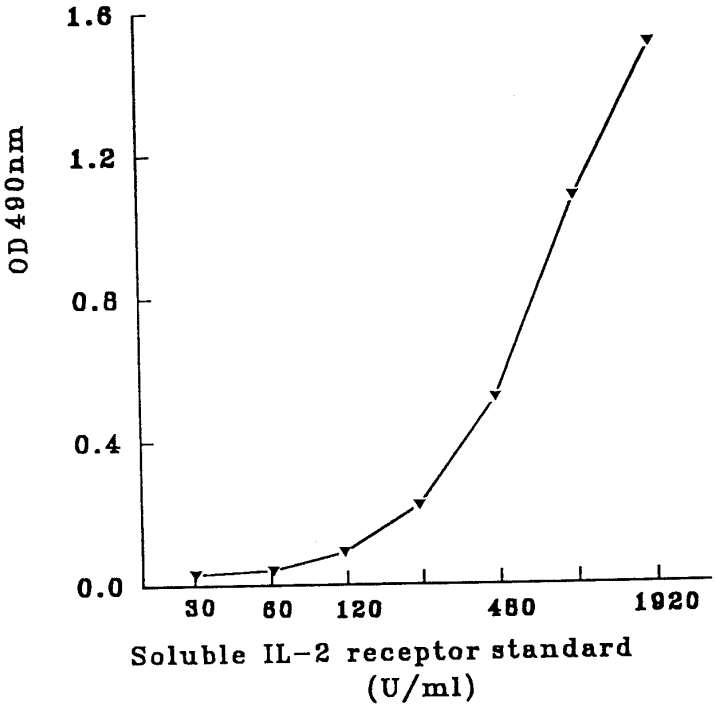


Figure 4.8. A standard curve of the soluble IL-2 receptor assay using a two-site ELISA. The plate was coated with MAb anti-sIL-2R (7G7B6) at a concentration of 5 µg/well. A biotinylated second monoclonal anti-sIL-2R (anti-TAC) antibody and streptavidin horseradish peroxidase-conjugated were used in the revealing step at

concentrations of 2 μg/ml and 1 μg/ml respectively. The plot represents the absorbances at 490 nm (vertical axis) versus the standard of sIL-2R at a range of dilutions from 30-1920 U/ml (horizontal axis).

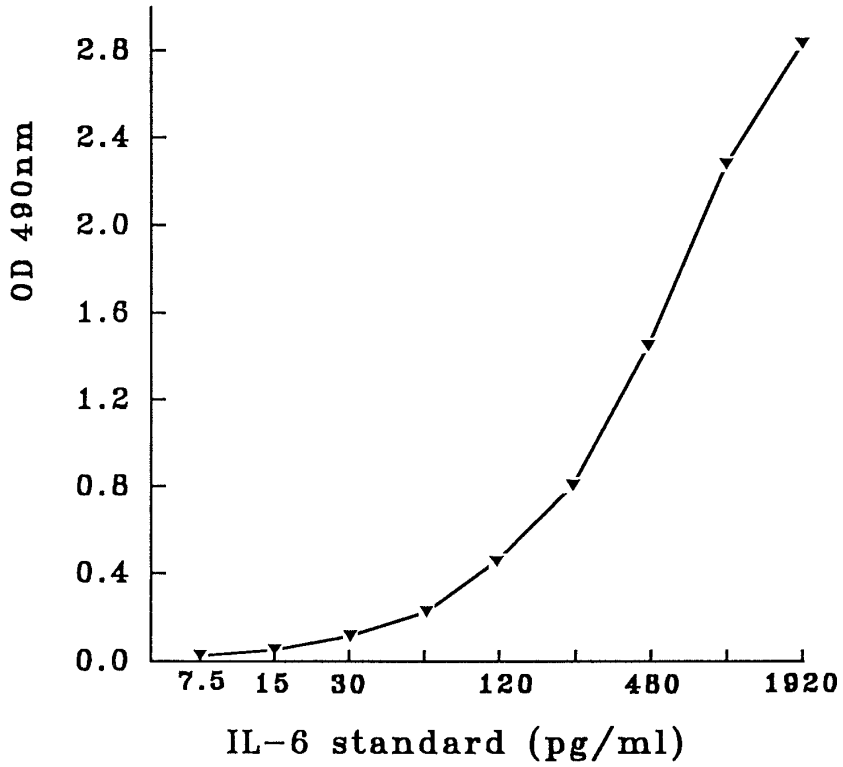


Figure 4.9. A standard curve of the IL-6 assay using a two-site ELISA. The plate was coated with anti-IL-6 MAb (clone CLB. IL-6/8) at a concentration of 1.5 μg/well. A biotinylated second anti-IL-6 (1G61) MAb and streptavidin horseradish peroxidase-conjugated were used in the revealing step at concentrations of 2 μg/ml and 1 μg/ml respectively. The plot represents the absorbances at 490 nm (vertical axis) versus the standard of IL-6 at a range of dilutions from 7.5-1920 pg/ml (horizontal axis).

Measurement of IL-4 and IL-10. The determination of IL-4 and IL-10 was performed according to the technique of two-site ELISA by Abrams et al. (1992) in which combinations of two monoclonal antibodies (clones 82 and 12 for IL-4 and JES3-9D7 and JES3-12G8 for IL-10) that recognise distinct epitopes of IL-4 and IL-10 were used in coating and revealing steps respectively. The antibodies used in the revealing step were biotinylated and the reaction was developed by addition of peroxidase-conjugated streptavidin.

IL-4 and IL-10 immunoassays were performed in similar conditions used for the IL-6 immunoassay except that: samples and standards were incubated overnight at 4°C instead of the 2 hour incubation time at room temperature. The optimum concentrations of coating and revealing antibodies and incubation times were determined as described previously for TNF-α immunoassay. The sensitivity of the assay was 10 pg/ml for IL-4 and 6 pg/ml for IL-10 (**Figures 4.10**). No cross reaction was observed with other cytokines such as IL-1α, IL-1β, TNF-α and IFN-γ. Inter-assay and intra-assay coefficient of variation (%) of test samples for both IL-4 and IL-10 were determined in a similar manner described in TNF-α immunoassay and ranged between 6%-8%.

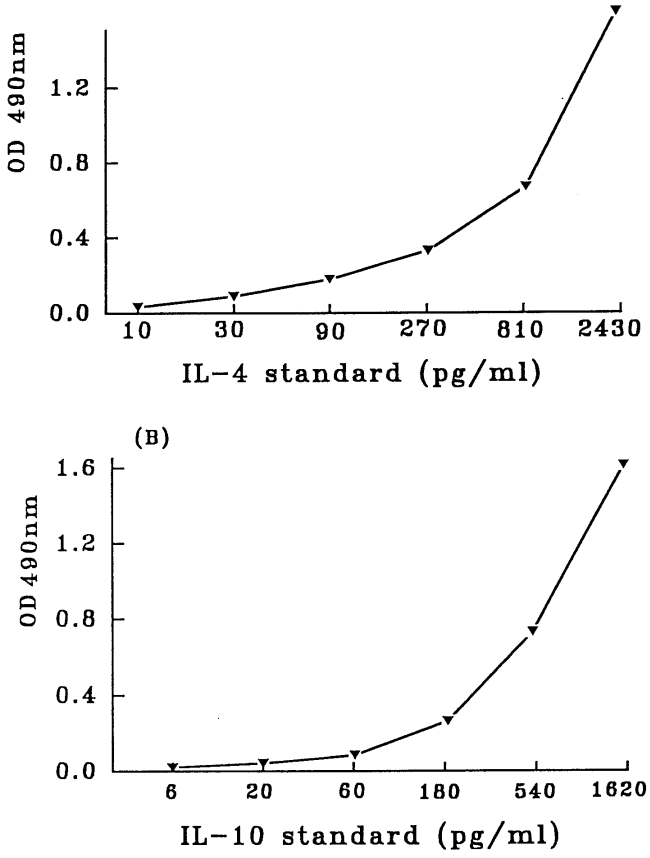


Figure 4.10. Standard curves of IL-4 (A) and IL-10 (B) using two-site ELISA. The plate was coated with MAb anti-IL-4 (clone 82) and anti-IL-10 (JES3-9D7) at a concentration of 1 μg/well for both. A biotinylated second MAb anti-IL-4 (clone 12) and anti-IL-10 (JES3-12G8) and streptavidin horseradish peroxidase-conjugated were used in the revealing step and all at concentrations of 1 μg/ml. The plot represents the absorbances at 490 nm (vertical axis) versus the standard of IL-4 at a

range of dilutions from 10-2430 pg/ml and IL-10 at a range of dilutions from 6-1620 pg/ml (horizontal axis).

4.6.2.2 IMMUNOASSAYS OF MOUSE CYTOKINES

Serum levels of mouse TNF-α, IL-2 and IL-4 were measured using two-site ELISA as described in section 4.6.2. Immunoassay of mouse TNF-α was established similarity to that for human TNF-α immunoassay using hamster anti-mouse TNF-α monoclonal antibody (clone TN3 19-12) in the coating step and sheep anti-mouse TNF-α polyclonal antibody in the revealing step. Immunoassays for the measurement of murine IL-2 and IL-4 were set up using combinations of two monoclonal antibodies (JES6-IA12 and JES6-5H4 clones for IL-2 and BVD4-1D11 and BVD6-2492 clones for IL-4) recognising distinct epitopes on the cytokines and were used in coating and revealing steps of IL-2 and IL-4 respectively. The antibodies used in the revealing step were biotinylated. The assay was assessed in similar manner to the human IL-4 immunoassay. The optimum concentrations of antibodies used in the immunoassay were determined as described above (vide 4.6.2.1).

The sensitivity of TNF-α, IL-2 and IL-4 was 10 pg/ml. No cross reaction was observed with mouse IL-1α and IFN-γ or human cytokines. Inter-assay and intra-assay coefficient of variation (%) of test samples ranged between 7%-12% for both IL-2 and IL-4, and 5%-11% for TNF-α.

4.6.2.3 CYTOKINE BIOASSAY

IL-2 bioassay. Levels of IL-2 activity in the serum and tissue culture supernatant were measured in a bioassay using an IL-2 dependent cell line (CTLL-16, murine cytotoxic T-cell line) (Gillis et al., 1978). This cell line was maintained in RPMI 1640 culture medium containing 10% foetal calf serum and supplemented with 100 U/ml recombinant human IL-2. Cell proliferation was assessed using a rapid and sensitive modified colorimetric method (Heeg et al., 1988), which is based on the ability of viable cells to cleave 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide MTT and can be measured on a scanning multiwell spectrophotometer (ELISA reader). The CTLL-16 cells were washed four times in Hank's balanced salt solution (HBSS) to remove any IL-2 contamination. The cells were then resuspended in RPMI 1640 medium at a concentration of $1x10^5$ cells/ml and 100µl of this suspension were pipetted into each well of flat-bottomed 96 well microtitre plates. This was followed by the addition of 100µl of the test sample (serial dilution

of test sera or supernatant) to each well in triplicate. After incubation for 20 hour at 37^{o}C/5% CO_2, 20µl of 2.5 mg/ml MTT was added to each well. After additional incubation at 37^{o}C for 4 hour, 100µl of acid isopropanol were added (to dissolve blue formazan crystals). The optical density was read in an ELISA reader using dual wavelength to remove the absorbance caused by interfering factors such as dirt, tissue culture debris, scratches and moisture in the plastic of microtitre plate. The test wavelength was set at 570 nm and the reference wave length set at 690. A typical sigmoid standard curve of the IL-2 bioassay is shown in **Figure 4.11**. The levels of IL-2 in test samples were derived using a known standard and calculating the percentage of maximal CTLL stimulation as described by Kay et al. (1986).

The percentage of maximal CTLL stimulation= 100x ([OD of maximal CTLL stimulation in test sample - background activity] divided [maximal CTLL stimulation in standard - background]).

TNF-α bioassay. To measure the biological activity of TNF-α levels in serum detected in the TNF-α immunoassay, a TNF-α bioassay was set-up using the L929 cell line cytotoxicity assay based on detachment of damaged cells from the wells (Meager et al., 1987). 100 µl RPMI culture medium containing 2x10^{5} trypsinized L929 cells were distributed in each well of a 96-well plate and incubated at 37^{o}C for 24 hours; titrations of the TNF standard and dilutions of the samples in duplicate in medium containing 1 µg/ml actinomycin **D** (to inhibit L929 cell line growth) were added to the cells and incubated at 37^{o}C for 24 hour; the cells were then washed, stained with 0.1% crystal violet for 10 min and fixed with 5% formalin. The absorbance at 620 nm was determined using an ELISA reader. The assay sensitivity for TNF-α was 0.3 U/ml and 1 U of TNF is equivalent to 38 pg.

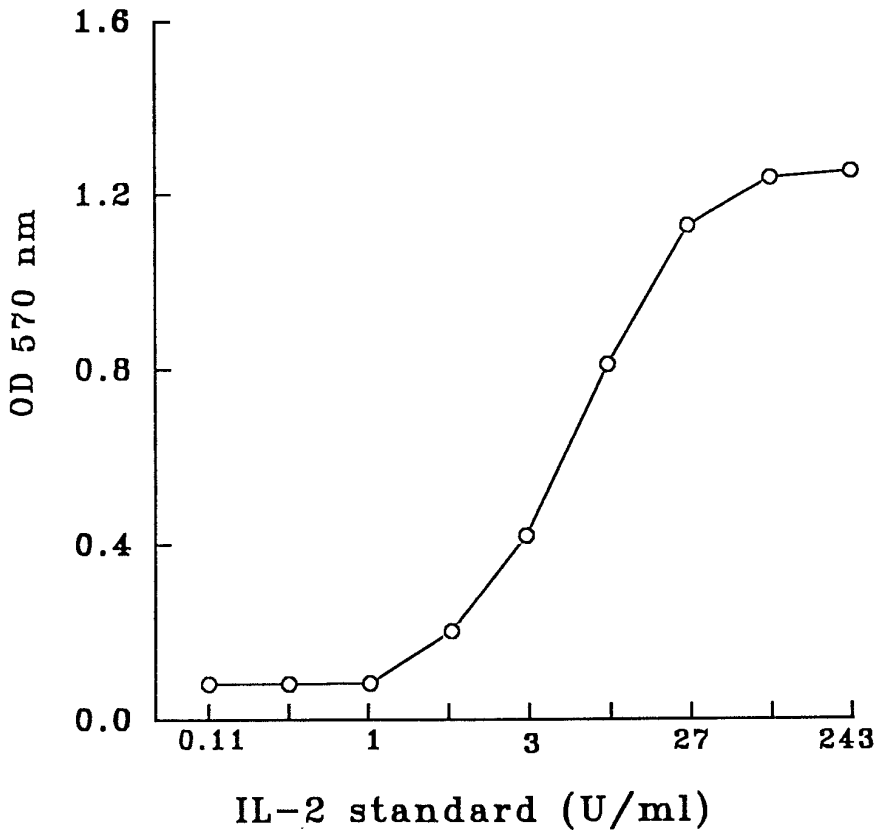


Figure 4.11. A standard curve of the IL-2 bioassay using IL-2 dependent cell line (CTLL-16) in a colorimetric microassay. The plot represents the optical density (OD) read in an ELISA reader using dual wavelength, with the test wave length set at 570 nm and the reference wave length set at 690 nm (vertical axis) versus the standard of IL-2 at a range of dilutions from 0.11-243 U/ml (horizontal axis).

4.6.3 MITOGEN-INDUCED SECRETION OF CYTOKINE

The majority of cytokines are not produced by peripheral blood mononuclear cells in isolation but usually together with mitogens (antigens) in vitro (Lewis, 1991). Phytoheamoagglutinine (PHA) was found to stimulate only T cells while Lipopolysaccharride (LPS) stimulates macrophages (Hudson and Hay, 1989). Induction of cytokine secretion from these two population mononuclear cells was investigated in RPMI 1640 culture medium supplemented with PHA or LPS. PHA was used for induction of IL-2 and IFN-γ (Karen et al., 1984) while LPS was used for induction of IL-1α and TNF-α secretion (Fomsgaard et al., 1990). Peripheral blood mononuclear cells (PBMC) were isolated from heparinised venous blood by sedimentation on a Ficoll-hypaque gradient, washed three times in HBSS and

resuspended at a concentration of 1 x 10^6/ml in RPMI 1640 culture medium. Cell viability was >90% as assessed by trypan-blue exclusion. PBMC were then divided into three tubes. The first and second tubes were treated with PHA and LPS respectively at concentration of 1μg/ml while the third tube contained PBMC only, without mitogens, as for control. After 24 hours of culture in humidified 95% air/5% CO_2 atmosphere, supernatants were collected, centrifuged, sterilized by filtration (0.22 μm pore size) and stored at -20^{o}C until tested.

Since there was no difference in the spontaneous secretion of cytokines and after mitogens-stimulation between healthy children and adults, and therefore, these were considered as one control group.

4.6.4 IMMUNOHISTOCHEMICAL AND IMMUNOFLUORESENT STAINING OF CYTOKINE PRODUCING CELLS IN NOD MOUSE PANCREAS SECTIONS

To investigate cellular expression of cytokine in sections of pancreas, immuno-histochemical and fluorescence staining was performed on cryostat pancreas sections from NOD mice with diabetes. The immunohistochemical staining was performed using two step immunoperoxidase technique in which peroxidase-conjugated anti-species immunoglobulin was used to label anti-cytokine monoclonal and polyclonal antibodies (Foulis et al., 1991). Indirect immunofluorescence was performed using fluorescein isothiocyanate (FITC)-conjugated rabbit anti-species immunoglobulin to visualise anti-cytokine polyclonal antibodies (Hudson and Hay, 1989).

Pancreas. Sections had been taken from pancreata removed from sacrificed NOD mice with various stages of disease progression. Pancreata were embedded in OCT (OCT compound) mounting medium immediately upon collection and stored in liquid nitrogen until use.

Immunological reagents. Monoclonal rat anti-mouse IFN-γ and polyclonal sheep anti-mouse TNF-α antibodies were employed for the detection of IFN-γ and TNF-α producing cells respectively in the pancreas sections of NOD mice. Peroxidase-conjugated rabbit anti-rat immunoglobulin and FITC labelled donkey anti-sheep antisera were the second reagents used to label monoclonal and polyclonal antibodies in the two-step immunoperoxidase and immunofluorescent techniques. Optimum concentration of immunological reagents used in these techniques were determined as described previously (vide section 4.6.2.1). **Table 4.5** shows the titres at which antibodies gave optimum staining performance and which were adopted in

the present study for use on murine pancreas sections.

Sectioning of pancreas. OCT embedded pancreata were removed from liquid nitrogen and quickly mounted on the chuck of a cryostat and allowed to reach -20^{o}C. 5 μm thick sections were placed onto slides, which had previously been cleaned with a concentrated solution of hydrochloric acid, rinsed in distilled water, coated with a 0.01% solution of poly-L-lysine (PLL) and allowed to dry. The use of PLL afforded two advantages: firstly, it retained the tissue section on the slide, and secondly by exerting a high surface tension, it prevented aqueous solutions applied to the slide from spreading. Two sections, one for each of the monoclonal or polyclonal antibodies to be used, including the control, were cut from each pancreas.

Fixation of pancreas sections. After cutting, pancreas sections were allowed to air-dry for 4-6 hours at room temperature. This ensures complete extraction of water from the sections, rendering them permeable to the fixative subsequently applied (Judd and Britten, 1982). Fixation was performed by immersion sections in a 1/1 mixture of acetone and chloroform for 10 minutes. This is a mild form of fixation, which achieves the best compromise between the need to preserve the tissue specimen and the need to avoid the chemical changes which alter its structure at the molecular level (Senaldi et al., 1991). After fixation, sections were washed in three changes of PBS of 5 min each.

STAINING OF PANCREAS SECTIONS

Detection of IFN-γ producing cells. Immunohistochemical staining of IFN-γ, was preceded by two preparatory steps: blocking of the endogenous peroxidase and activity blocking of non-specific binding of immunological reagents.

To block endogenous peroxidase activity, sections were covered with 1% solution of H_2O_2 in methanol for 20 min in a humidified atmosphere. This enzyme is present in large amounts in organs which are metabolically active such as the pancreas and may lead to high background levels of staining through its interaction with the reaction substrate (Khan et al., 1990). Sections were then washed and in order to block non-specific binding were incubated for 10 min with PBS containing 10% normal rabbit serum. The use of rabbit serum in this step prevents non-specific binding of the peroxidase-conjugated antiserum, which is raised in rabbit (Straus, 1979). After discarding the rabbit serum, the sections were immediately incubated for 1 hour with a 1/10 dilution of rat anti-mouse IFN-γ monoclonal antibodies. Sections were then washed again and incubated with a dilution of 1/40 peroxidase-conjugated rabbit anti-rat immunoglobulin in PBS, containing 5% normal mouse

serum. After incubation, sections were washed, immersed for 8 min in a solution of 0.6 mg/ml diaminobenzidine hydrochloride and 0.03% H_2O_2 in PBS, rinsed in tap water, counterstained with Mayer's haematoxylin, washed again extensively in tap water and finally mounted with a 9/1 mixture of glycerol and PBS.

The specificity of the staining was determined by prior incubation of anti-mouse IFN-γ antibodies with 100 μg/ml of recombinant mouse IFN-γ which resulted in abolition of the peroxidase staining.

Detection of anti-TNF-α producing cells. An Immunohistochemical technique was used to detect TNF-α producing cells in pancreas sections using polyclonal anti-TNF-α antibodies under the same conditions used for the detection of IFN-γ. Although this technique is very sensitive, a number of difficulties emerged. The positive staining of TNF-α producing cells was weak and there was some non-specific background staining due to endogenous peroxidase activity. The weakness of positive TNF-α staining in pancreas may be due either to the low expression of TNF-α or to the destruction of the antigen during the technique used to block the endogenous peroxidase activity. Therefore, immunohistochemical staining was replaced by an indirect immunofluorescent technique which was then attempted for detection of TNF-α producing cells in pancreas sections.

5μm air-dried, fixed cryostat sections of pancreas were incubated with 50 μl sheep anti-mouse TNF-α diluted in 1/100 in PBS for 60 minutes at room temperature. Following two 10-minute washes in PBS, TNF-α was revealed using FITC labelled anti-sheep immunoglobulin G diluted 1:30 in PBS. After washing, the sections were examined using an ultraviolet (UV) microscope. This technique proved to be successful.

Microscopic analysis. Stained and unstained mononuclear cells in three islets of β-cells were counted using light microscopy for IFN-γ producing cells while a UV microscope was used to count TNF-α positive cells. The number of positive cells was calculated and expressed semiquantitatively, with 0 equalling no positive cells, 1 minimal (<10%), 2 moderate (10-30%) and 3 abundant (>30%) quantities of positive cells.

4.6.5 IMMUNOHISTOCHEMICAL STAINING OF CYTOKINE PRODUCING CELLS IN LIVER BIOPSIES

To investigate the cellular expression of cytokine in the liver biopsies, we immunohistochemically stained cryostat liver sections from children with autoimmune liver disease. The mononuclear cells (MNC) infiltrate was

characterised in liver sections from 36 such biopsies.

Liver biopsies. Biopsies were taken percutaneously with a 1.4 mm diameter Menghini needle and consisted of 3-5 mm long liver tissue cylinders. Only biopsy material in excess of the amount required for diagnostic examination was employed in this study. Biopsies were embedded in OCT medium immediately upon collection and stored in liquid nitrogen until use.

Immunological reagents. Two murine MAb were employed for the detection of TNF-α and IFN-γ producing cells. They are anti-human TNF-α (clone 357-27) and anti-human IFN-γ (clone M23B5) monoclonal antibodies. Peroxidase-conjugated rabbit anti-mouse immunoglobulin antiserum was the revealing reagent.

Staining of liver sections. Positive staining of TNF-α and IFN-γ producing cells was detected in liver sections using the two step indirect immunoperoxidase technique performed in a similar manner to that for IFN-γ staining in pancreas sections of NOD mice (vide 4.6.4).

The specificity of the staining was determined by prior incubation of detector antibodies such as anti-TNF-α and anti-IFN-γ antibodies with 100 μg/ml of recombinant TNF-α and IFN-γ respectively which resulted in abolition of the peroxidase staining.

Microscopic analysis. Stained and unstained mononuclear cells in one to three portal tracts were counted using light microscopy and scored as positive or negative in a blind study. The percentage of positive cells was calculated and expressed semiquantitatively, with 0 equalling no positive cells, 1 minimal (<5%), 2 moderate (5-10%) and 3 abundant (>10%) quantities of positive cells.

4.6.6 METABOLIC STUDIES

The metabolic disturbances associated with diabetes have been reported to influence immune function (Selam et al., 1979; Crosti et al., 1986). In order to be able to control for this, it is important to assess the degree of metabolic disturbance. Typically, this is achieved by measuring blood glucose and glycated haemoglobulin (HbA_1) at the time of blood sampling. Blood glucose is an indicator of current metabolic control; HbA_1 reflects metabolic control over the previous weeks (Kjaergaard and Ditzel, 1979).

Blood glucose levels were measured in the blood of patients with Type 1 diabetes and discordant twins for diabetes using a glucose-oxidase method (Yellow Springs Analyser, Yellow Springs, OH, USA) and HbA_1 by electroendosmosis (Corning Instruments, Corning, NY, USA).

4.6.7 C-PEPTIDE LEVELS

Human C-peptide and insulin are formed from proinsulin, the precursor of endogenous insulin in the β-cells of the pancreas. Both proteins are released into the bloodstream simultaneously and in equimolar quantities. The measurement of human C-peptide provides an indicator of the residual synthetic capacity of β-cells. Serum levels of C-peptide were measured in patients with Type 1 and Type 2 diabetes using a competitive radioimmunoassay (Heding, 1975). Assay sensitivity was 0.3 ng/ml, intra-and inter-assay coefficients of variation were 6.5% and 13% respectively.

4.6.8 DETECTION OF ORGAN-SPECIFIC AND NON ORGAN-SPECIFIC AUTOANTIBODIES

Cytoplasmic islet cell antibodies. Islet cell cytoplasmic antibodies (ICA) were detected in a two-step indirect immunofluorescence assay according to the technique of Bottazzo et al., (1979). Unfixed 5μm cryostat sections of blood group O human pancreas were used as substrate. 50 μl neat sera were incubated with the substrate for 30 minutes at room temperature. Following two 10-minute washes in PBS, ICA were revealed using FITC anti-human immunoglobulin G $F(ab')_2$ fragment diluted 1:30 in PBS. Following two washes slides were viewed by UV microscopy. The results obtained are reported as positive (level >5 JDF [Juvenile Diabetes Foundation] units) or negative. The method of detection was similar to that performed in the stage III Immunology and Diabetes Workshop on ICA proficiency (Bonifacio et al., 1987), performing with 100% specificity, a detection limit of 5 JDF units and with a correlation of observed with consensus JDF units of 0.74.

Glutamic acid decarboxylase antibodies. Antibodies to glutamic acid decarboxylase (GAD) were measured by determining the enzyme activity immunoprecipitated by sera from a soluble extract of rat brain as previously described (Christie et al., 1992) and are reported here as positive (levels >2SD above normal values for our laboratory) or negative. In the first GAD antibody workshop our assay performed with a specificity of 100% and a sensitivity of 91.7%.

Liver-kidney microsomal antibodies. Anti-liver-kidney microsomal (anti-LKM) antibodies were detected by two-step indirect immunofluorescence technique on unfixed 5 μm cryostat sections of rat liver, stomach and kidney as the substrate according to the technique of Smith et al., (1974). 50 μl of test sera diluted 1/10 in

PBS were incubated with the substrate and the pattern of positive staining of anti-LKM antibodies is described in **Table 4.10**.

Non organ-specific autoantibodies detected by immunofluorescence. Non-organ specific autoantibodies (anti-nuclear (ANA), anti-smooth muscle (SMA), anti-gastricparietal cell (GPC), anti-mitochondrial (AMA) and anti-reticulin (ARA) antibodies were detected in a serum by a technique similar to that mentioned in section 4.12.3 using rat liver, kidney and stomach as substrate (Johnson and Holborrow, 1986). The pattern of positive staining for these autoantibodies is described in **Table 4.10**.

Table 4.10 Patterns of positive staining of non-organ specific autoantibodies by two step indirect immunofluorescence technique.

Auto-antibodies*	**Patterns**
ANA	Nucleic staining in all tissues
SMA	Smooth muscle staining was seen in the sub-mucosal muscle layer of the stomach in conjunction with the staining of smooth muscle fibres in the gastric mucosa. Blood vessels in both kidney and liver are also stained and in high titre SMA the glomeruli are also stained.
AMA	Liver cytoplasm stained with a speckled appearance Gastric cells also stained similarly, the parietal cells being especially strong Kidney tubules stained to varying degrees depending on whether proximal or distal.
LKM	Liver cytoplasm stained uniformly. No staining in stomach Only the proximal tubules are stained the pattern being a slightly "feathery" appearance compared to a AMA staining.
GPC	Only the cytoplasm of the parietal cells of the stomach are stained with no involvement of other tissues.
ARA	"Thread-like" linear staining in gastricmucosa

***** Non-organ specific autoantibodies; ANA, anti-nuclear; SMA, anti-smooth muscle, AMA, anti-mitochondrial; LKM, anti-liver-kidney-microsomal; GPC, anti-gastricparietal cell; ARA, anti-reticulin (ARA)

Partical agglutination technique for thyroid autoantibodies. Autoantibodies reacting with thyroid microsomal antigens and with thyroglobulin were detected by particle agglutination using commercially available kits. In these, sensitised gelatin particles are incubated with serial dilutions of serum in the wells of round-bottomed microtitre plates. After 3 hours, positive results appear as a carpet of particles in the wells, negative as a tight button.

4.6.9 HLA TYPING

Class II HLA typing by Restriction Fragment Length Polymorphism Analysis.
Class II HLA typing, performed using restriction fragment length polymorphisms (RFLP) (Doherty and Donaldson, 1991) on cryopreserved PBMN, was kindly carried out by Dr P. Donaldson, Institute of Liver Studies, King's College School of Medicine and Dentistry. HLA-DR B gene polymorphism were identified using a 517 bp PstI fragment of the exon-specific HLA-DR B cDNA clone pRTv1 (Bidwell and Jarrold, 1986). HLA-DR B allogenotypes were identified from the hybridization patterns visualised by autoradiography (Bidwell and Jarrold, 1986; Bidwell, 1988).

4.6.10 STATISTICAL ANALYSIS

The distribution of the concentrations of cytokines and sIL-2 receptor did not satisfy the hypothesis of normality where this was assessed using the Kolmogorov-Smirnov goodness of fittest and these were therefore compared using the non-parametric Mann-Whitney U test. Correlation between variables were analysed by calculating Pearson's correlation coefficient or performing Spearman's rank correlation tests as appropriate. The frequency distribution of abnormal levels of cytokines in prediabetic and non-diabetic twins at least one and two occasions was compared using χ^2. Positively-stained cytokine-producing cells occurring in different patient group was also compared using χ^2.
Analyses giving p values of <0.05 were considered statistically significant.
Statistical computation were performed using the Statistical Package for the Social Sciences on the University of London Computer Centre's Amdah l5980/300.

CHAPTER 5

STUDIES OF CIRCULATING LEVELS OF CYTOKINES IN PATIENTS WITH TYPE 1 DIABETES

5.1 SUBJECTS

Thirty one recently diagnosed patients with Type 1 diabetes (17 males; median age 23 years, range 9-58) were studied. Seventeen of them were tested at diagnosis and before insulin-treatment, of the remainder 3 were tested within 2 months of diagnosis and the remaining 11 between 4 and 6 months after diagnosis. Type 1 diabetes was diagnosed according to the National Diabetes Data Group (NATIONAL DIABETES DATA GROUP, 1979). A group of 32 patients with long-standing Type 1 diabetes (10 males; 39 years, 24-75), who had been diagnosed for more than 15 years was also studied.

As disease controls, two groups of patients were studied: (1) to allow for the effect of hyperglycaemia we tested 27 patients with Type 2 diabetes seen consecutively at the diabetic clinic at King's College Hospital (14 males; median age 64 years, range 38-77; (2) to allow for the effects of a chronic autoimmune disease, 21 patients with Graves' disease were studied but without diabetes, seen consecutively at King's College Hospital during the same period as the collection from diabetic patients (9 males; median age 40 years, range 18-67). As normal control subjects, 41 healthy individuals (20 males; mean age 24 years, range 10-63) were studied. They were recruited from the local community and selected to achieve a similar distribution for age and sex to the twins and patients with diabetes. Twenty-four were adults recruited from the staff of King's College Hospital and 17 were healthy children recruited by the Medway and Gillingham Branch (Kent) of the Children's Liver Disease Foundation (CLDF). All gave blood after informed consent from themselves or their parents, as appropriate. None of the control subjects had a family history of Type 1 diabetes or other diseases.

At the time of sampling, neither control subjects nor the patients with Type 1 or Type 2 diabetes had clinical signs or symptoms of intercurrent illness.

5.2 RESULTS

Serum levels of IL-1α, IL-1β, IL-6 and TNF-α (macrophage-derived cytokines), IL-2 and IFN-γ (T_H1-derived cytokines), IL-4 and IL-10 (T_H2 derived-cytokines) and sIL-2R were measured in patients with Type 1 diabetes of different disease duration.

5.2.1 MACROPHAGE-DERIVED CYTOKINES

TNF-α. Levels of TNF-α were significantly higher in patients with recently diagnosed Type 1 diabetes (median, 29 pg/ml, range 0-434; $p<0.001$), long-standing

Type 1 diabetes (17 pg/ml, 0-110; $p<0.005$) and Type 2 diabetes (7 pg/ml, 0-75; $p<0.01$) when compared with normal control subjects (0 pg/ml, 0-9). Levels of TNF-α were significantly higher in patients with recently diagnosed Type 1 diabetes as compared with patients with either long-standing Type 1 ($p<0.04$), Type 2 diabetes ($p<0.005$) or Graves' disease ($p<0.0002$). Of patients with recently diagnosed Type 1 diabetes, levels of TNF-α were higher in those tested before insulin treatment (59 pg/ml, 0-434) than in those tested a median of 4 months later (19 pg/ml, 0-54; $p<0.01$). Levels of TNF-α were higher in patients with long-standing Type 1 diabetes than in those with Graves' disease ($p<0.001$) and in those with Type 2 diabetes although in the latter case the increase did not reach conventional levels of statistical significance ($p<0.07$). Levels of TNF-α exceeding the highest normal value (>9 pg/ml) were found in 23/31 (74%) recently diagnosed patients and 18/32 (56%) long-standing patients with Type 1 diabetes; 6/27 (22%) patients with Type 2 diabetes, and 6/21 (28%) patients with Graves' disease (**Figure 5.1**).

Patients who had levels of TNF-α exceeding the highest normal value and whose serum was available were also tested for TNF-α bioactivity. Bioactive TNF-α was detected in: 14/17 (88%) patients with recently diagnosed Type 1 diabetes (median 5 U/ml, range 0-18), 11 of whom had levels of TNF-α >1 U/ml; 6/17 (35%) long-standing patients with Type 1 diabetes (median 0 U/ml, 0-18), 3 of whom had levels of TNF-α >1 U/ml; and 2/11 (18%) patients with Type 2 diabetes (median 0 U/ml, 0-18), both having levels more than 1 U/ml. Levels of bioactive TNF-α correlated significantly with levels of TNF-α obtained by immunoassay ($r=0.65$, $p<0.003$) in patients with recently diagnosed Type 1 diabetes while this correlation was not observed in patients with long-standing Type 1 diabetes and Type 2 diabetes.

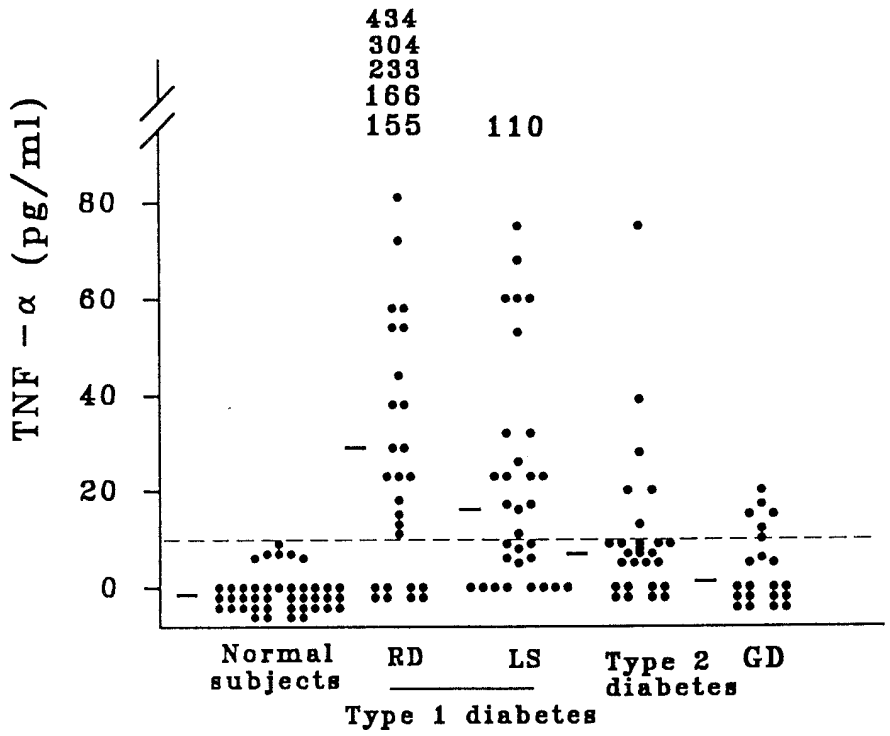


Figure 5.1. TNF-α values in the serum of patients with Type 1 diabetes of different duration, Type 2 diabetes, Graves' disease (GD) and normal subjects. The dashed line represents the upper limit of normal subjects. Levels of TNF-α are significantly higher in recently diagnosed (RD) ($p<0.001$) and long-standing (LS) patients with Type 1 diabetes ($p<0.005$) and Type 2 diabetes ($p<0.01$) than normal subjects. Horizontal bars represent medians.

IL-1α. Levels of IL-1α were significantly elevated in patients with recently diagnosed Type 1 diabetes (median 260 pg/ml, range 59-1421 pg/ml) compared with patients with either long-standing Type 1 (129 pg/ml, 30-303; $p<0.001$) or Type 2 diabetes (105 pg/ml, 29-402; $p<0.005$), Graves' disease (84 pg/ml, 12-480; $p<0.001$) or normal control subjects (100 pg/ml, 23-233; $p<0.001$) (**Figure 5.2**). Of patients with recently diagnosed Type 1 diabetes, those tested before insulin treatment (260 pg/ml, 61-521) had similar levels to those tested later (248 pg/ml, 59-1421). Levels of IL-1α exceeding the highest normal value (>233 pg/ml) were found in 18/31 (58%) recently diagnosed, 4/32 (13%) patients with long-standing Type 1 diabetes, 5/27 (19%) patients with Type 2 diabetes and 7/21 (33%) patients with Graves' disease. Levels of IL-1α did not differ significantly between patients with long-

standing Type 1 and those with Type 2 diabetes and Graves' disease, or between either of these groups and control subjects.

IL-1β and IL-6. Levels of IL-1β and IL-6 were not detectable in the serum of either patients or normal control subjects.

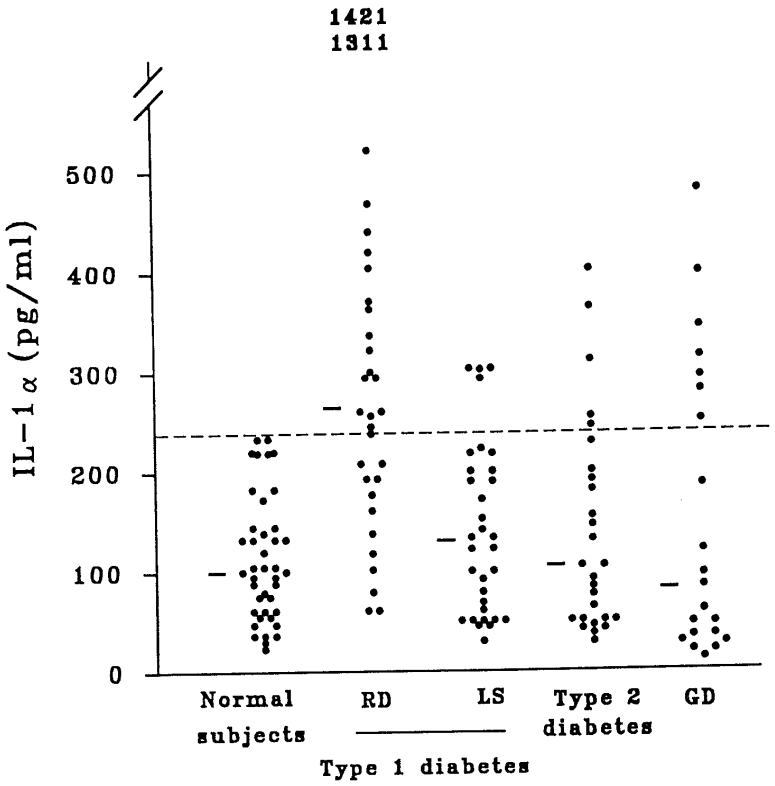


Figure 5.2. IL-1α values in the serum of patients with Type 1 diabetes of different duration, Type 2 diabetes, Graves' disease (GD) and normal subjects. The dashed line represents the upper limit of normal subjects. Levels of IL-1α are significantly higher in recently diagnosed (RD) patients with Type 1 diabetes than in patients with long-standing (LS) Type 1 diabetes ($p<0.01$), Type 2 diabetes ($p<0.005$), Graves' disease ($p<0.001$) and normal subjects ($p<0.001$). Horizontal bars represent medians.

5.2.2 T_H1 PROFILE

IL-2. Levels of IL-2 were significantly elevated in patients with recently diagnosed Type 1 diabetes (median 12 percent; range 3.7-56.0; $p<0.0001$), long-standing Type 1 diabetes (8 percent; 3.2-30.5; $p<0.001$), Type 2 diabetes (6.4 percent, 1-21.0;

p<0.001) and Graves' disease (6.3 percent, 2.2-15; p<0.01) when compared with normal control subjects (3.6 percent, 1-8) (**Figure 5.3**).

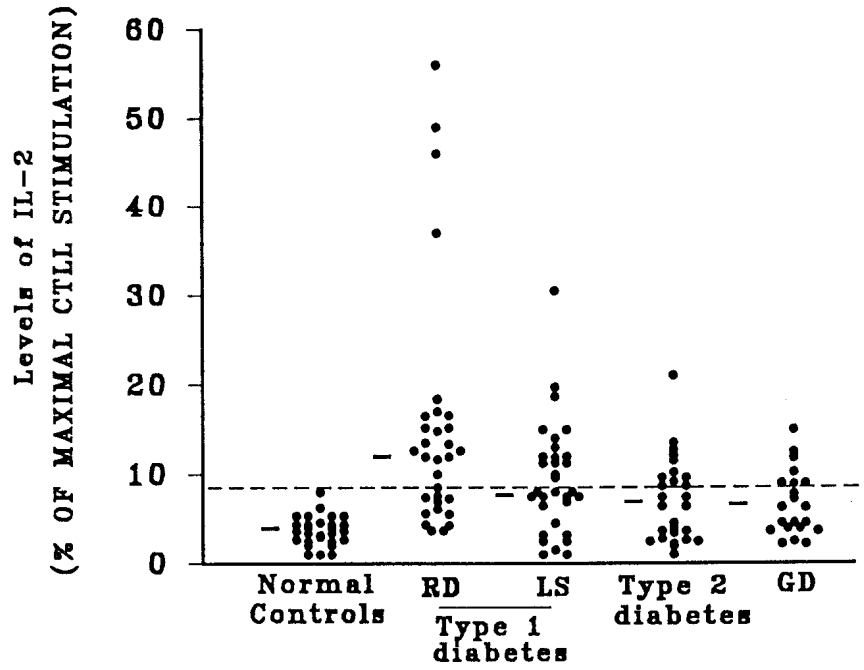


Figure 5.3. IL-2 values in the serum of patients with Type 1 diabetes of different duration, Type 2 diabetes, Graves' disease (GD) and normal subjects. The dashed line represents the upper limit of normal subjects. IL-2 levels were measured in a bioassay using an IL-2 dependent cell line, cytotoxic T-cell lymphocytes (CTLL), by a colorimetric method. The results are expressed as the percentage of maximal stimulation of CTLL in test samples. Horizontal bars represent medians. Levels of IL-2 are significantly higher in patients with recently diagnosed (RD) (p<0.0001) and long-standing (LS) Type 1 diabetes (p<0.001), Type 2 diabetes (p<0.001) and Graves' disease (p<0.01) when compared with normal subjects.

Levels of IL-2 were significantly higher in patients with recently diagnosed Type 1 diabetes when compared with patients with either Type 2 diabetes (p<0.01) or Graves' disease (p<0.003). Levels of IL-2 were not significantly different in those

tested before insulin treatment (7.6 percent, 3.5-56) and those tested later (12 percent, 4.3-49). Levels of IL-2 did not differ significantly between patients with long-standing Type 1 diabetes, Type 2 diabetes and Graves' disease. Levels of IL-2 exceeding the highest normal value (>8 percent) were found in 20/31 (64%) recently diagnosed patients and 15/32 (47%) long-standing patients with Type 1 diabetes; 11/27 (41%) patients with Type 2 diabetes, and 7/21 (33%) patients with Graves' disease.

IFN-γ. Levels of IFN-γ were significantly higher in patients with recently diagnosed Type 1 diabetes (median 225 pg/ml, range 0-1500) compared with patients with long-standing Type 1 diabetes (0 pg/ml, 0-435; $p<0.001$), Type 2 diabetes (0 pg/ml, 0-365; $p<0.001$), Graves' disease (0 pg/ml, 0-304; $p<0.005$) and normal control subjects (0 pg/ml, 0-130; $p<0.0001$) (**Figure 5.4**).

In patients with recently diagnosed Type 1 diabetes, levels of IFN-γ were not significantly different in those tested before insulin treatment (405 pg/ml, 0-1310) and those tested later (95 pg/ml, 0-1500). Levels did not differ significantly between patients with long-standing Type 1, Type 2 diabetes and Graves' disease, or between either of these groups and control subjects. Levels of IFN-γ exceeding the highest normal value (>130 pg/ml) were found in 18/31 (58%) recently diagnosed patients and 4/32 (13%) long-standing patients with Type 1 diabetes, 3/27 (11%) with Type 2 diabetes and 2/21 (9%) with Graves' disease.

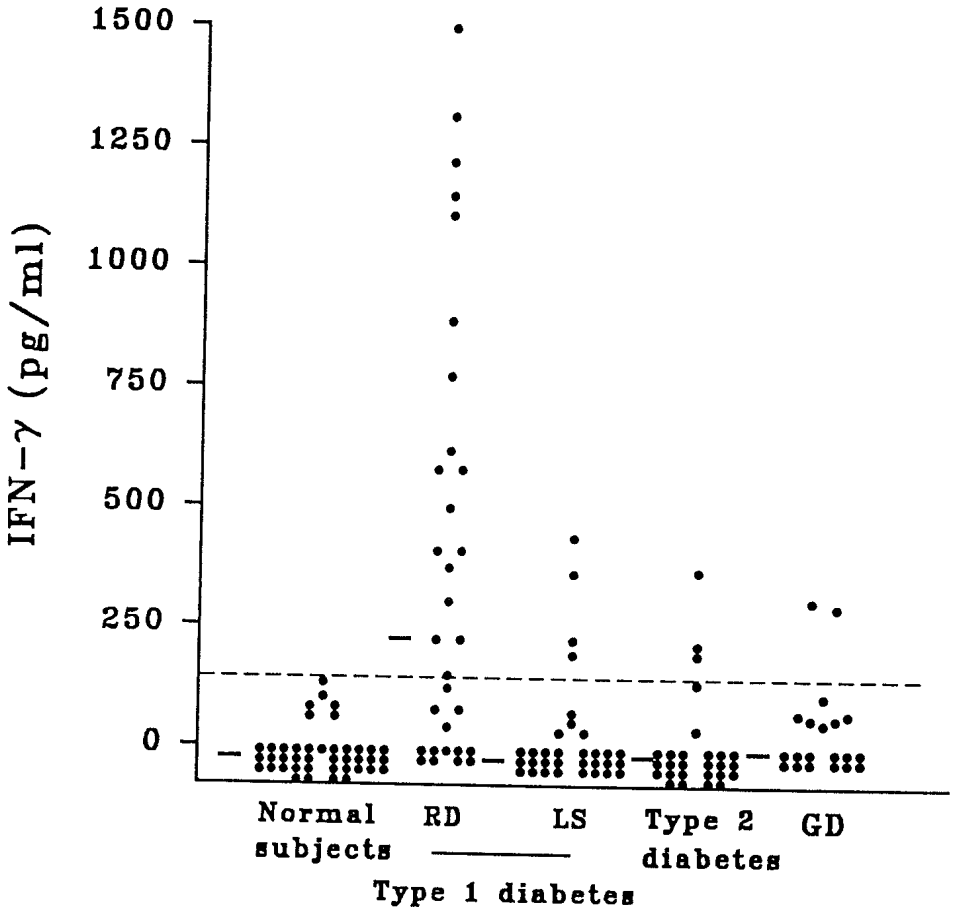


Figure 5.4. IFN-γ values in the serum of patients with Type 1 diabetes of different duration, type 2 diabetes, Graves' disease (GD) and normal subjects. The dashed line represents the upper limit of normal subjects. Levels of IFN-γ were significantly higher in recently diagnosed (RD) patients with Type 1 diabetes than patients with long-standing (LS) Type 1 diabetes ($p<0.001$), Type 2 diabetes ($p<0.001$), Graves' disease ($p<0.005$) and normal subjects ($p<0.0001$). Horizontal bars represent medians.

5.2.3 T_H2 PROFILE

IL-4 and IL-10. Median levels of IL-4 and IL-10 were not significantly different in patients with recently diagnosed and long-standing Type 1 diabetes, patients with Type 2 diabetes and normal subjects (**Figures 5.5A and B**). Significantly elevated levels of IL-4 and IL-10 were present in patients with Graves' disease when compared with recently diagnosed and long-standing patients with Type 1 diabetes,

patients with Type 2 diabetes and normal subjects ($p<0.001$ for all). Levels of IL-4 and IL-10 exceeding the highest normal value (42 pg/ml for both IL-4 and IL-10) were found in 3/31 (9%) and 1/31 (3%) recently diagnosed patients with Type 1 diabetes, 4/32 (10%) and 0/32 (0%) long-standing patients with Type 1 diabetes; 1/27 (3%) and 0 /27 (0%) patients with Type 2 diabetes, and 6/21 (28%) and 15/21 (70%) patients with Graves' disease respectively. Levels of IL-6 were not detectable in the serum of either patients or normal control subjects.

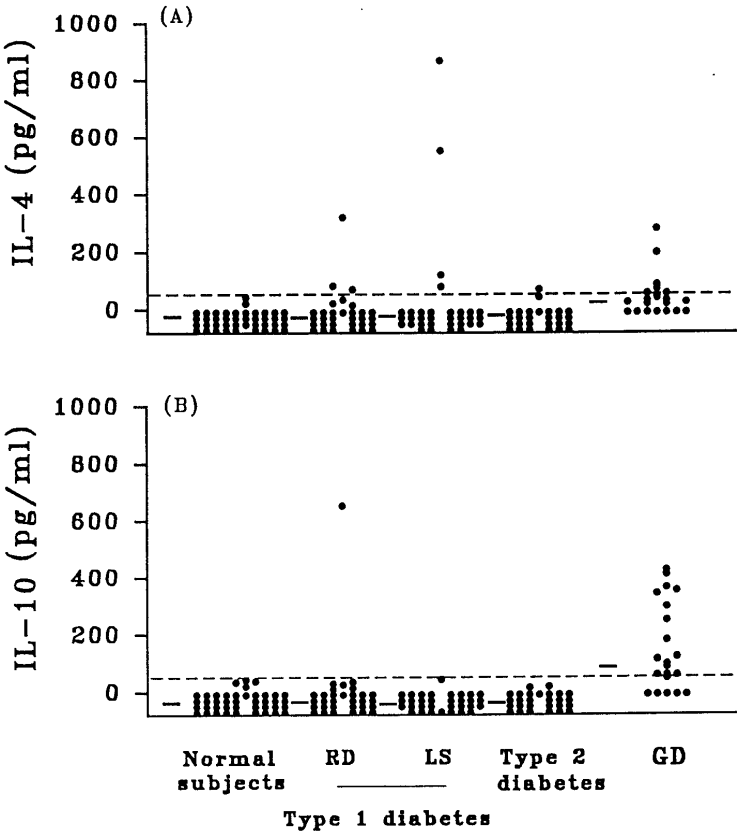


Figure 5.5. IL-4 (A) and IL-10 (B) values in the serum of patients with Type 1 diabetes of different duration, type 2 diabetes, Graves' disease (GD) and normal subjects. The dashed line represents the upper limit of normal subjects. Levels of IL-4 and IL-10 were not significantly different in patients with recently diagnosed (RD) and long-standing (LS) Type 1 diabetes, patients with Type 2 diabetes and normal subjects. Significantly elevated levels of IL-4 and IL-10 were present in patients with Graves' disease when compared with recently diagnosed and long-standing patients with Type 1 diabetes, patients with Type 2 diabetes and normal subjects ($p<0.001$ for all). Horizontal bars represent medians.

5.2.4 LEVELS OF sIL-2R IN TYPE 1 DIABETES

Levels of sIL-2R were significantly higher in patients with recently diagnosed Type 1 diabetes (median 384 U/ml, range 150-954) when compared with long-standing Type 1 diabetes (290 U/ml, 153-520; $p<0.02$), Type 2 diabetes (249 U/ml, 96-753; $p<0.03$), Grave's disease (252 U/ml, 100-760; $p<0.03$) and normal subjects (246 U/ml, 93-375; $p<0.001$) (**Figure 5.6**). Levels did not differ significantly between patients with long-standing Type 1, Type 2 diabetes, Graves' disease and normal controls. Levels of sIL-2R exceeding the highest normal value (375 U/ml) were found in 16/31 (51%) recently diagnosed patients and 9/31 (28%) long-standing patients with Type 1 diabetes, 4/23 (17%) patients with Type 2 diabetes and 5/20 (25%) patients with Graves' disease.

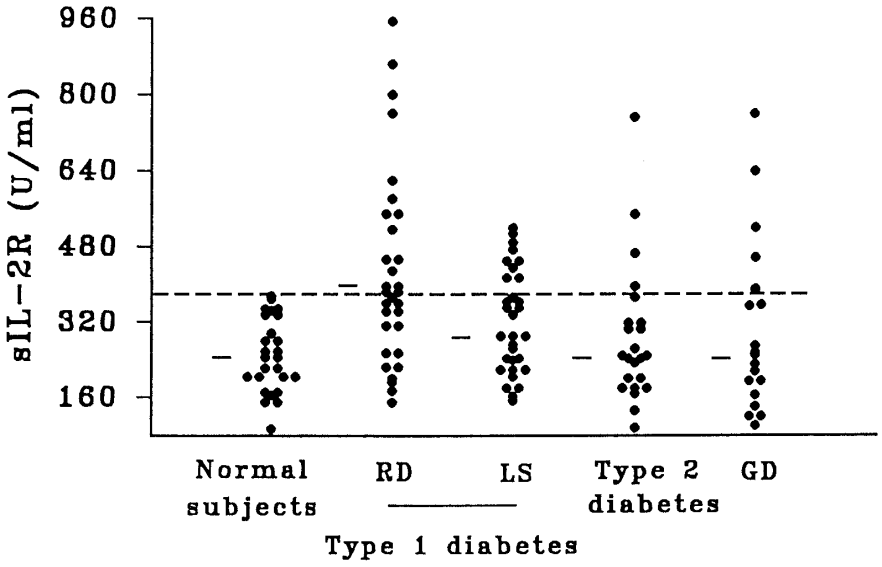


Figure 5.6. sIL-2R values in the serum of patients with Type 1 diabetes of different duration, Type 2 diabetes, Graves' disease (GD) and normal subjects. The dashed line represents the upper limit of normal subjects. Horizontal bars represent medians. Levels of sIL-2R are significantly higher in recently diagnosed (RD) patients with Type 1 diabetes than patients with long-standing Type 1 diabetes ($p<0.02$), Type 2 diabetes ($p<0.03$), Graves' disease ($p<0.03$) and normal subjects ($p<0.001$).

5.2.5 ASSOCIATION BETWEEN CYTOKINE LEVELS, sIL-2R, BLOOD GLUCOSE VALUES AND ISLET CELL AND GAD ANTIBODIES IN SUBJECTS STUDIED.

Levels of IFN-γ and TNF-α were significantly correlated in patients with recently diagnosed Type 1 diabetes ($r=0.51$, $p<0.05$), while levels of IL-1α and TNF-α were significantly correlated in patients with long-standing Type 1 diabetes ($r=0.41$, $p<0.05$). None of the other cytokines or sIL-2R were correlated with each other in any group studied.

Mean (± SD) blood glucose levels of patients with Type 1 and Type 2 diabetes at sampling were 13.2 ± 4.3 mmol/l and 11.2 ± 2.9 mmol/l respectively. No correlation was found between TNF-α, IL-1α, IL-2, IL-4, IL-10, IFN-γ or sIL-2R levels and blood glucose values of patients with Type 1 or Type 2 diabetes.

ICA and GAD antibodies were detected in 24/31 (78%) and 12/31 (39%) recently diagnosed patients respectively and in 6/32 (19%) and 3/32 (13%) long-standing patients with Type 1 diabetes respectively. All patients with Type 2 diabetes were negative for ICA and 4 of 27 (11%) were positive for GAD antibodies. In patients with Type 1 diabetes, there was no correlation between those whose levels of cytokines or sIL-2R exceeded the upper limit of normal and the presence of either ICA or GAD antibodies.

5.2.6 ASSOCIATION BETWEEN LEVELS OF CYTOKINES, sIL-2R AND C-PEPTIDE LEVELS.

As expected, median C-peptide levels were reduced in patients with recent onset and long-standing Type 1 diabetes (median 1.1, range 0.5-3.4 ng/ml and 0.77, 0.39-1.75 ng/ml respectively) compared with patients with Type 2 diabetes and normal controls (2.68, 1.0-6.4 ng/ml and 1.9, 1.6-5.4 ng/ml respectively). There was a significant correlation between C-peptide and TNF-α levels in patients with Type 1 diabetes of recent onset ($r=0.42$, $p<0.05$) as well as in those with long-standing Type 1 diabetes ($r=0.51$, $p<0.05$). There was no relationship between C-peptide levels and levels of IL-1α, IL-2, IL-4, IL-10, IFN-γ and sIL-2R.

5.3 COMMENT

Increased levels of TNF-α, IL-1α, IL-2 and IFN-γ - were found in recently diagnosed patients with Type 1 diabetes when compared with both disease and metabolic control subjects and with normal controls. The presence of this profile of cytokines implies activation of the T_H1 subset of helper cells near to diagnosis of Type 1 diabetes. Interferon-γ, released by this subset of T lymphocytes, is a potent stimulator of macrophage function, and the elevated levels of macrophage-derived cytokines TNF-α and IL-1α at diagnosis could be secondary to the T_H1 response. In contrast, no difference in the levels of IL-4 and IL-10 was observed between diabetic and normal control subjects, indicating a lack of recruitment of T_H2 subset at diagnosis of Type 1 diabetes.

It has been proposed that the balance of T_H1 and T_H2 cells is critical in the development of Type 1 diabetes (Harrison et al., 1993). This suggestion is made on the basis that at-risk first degree relatives who have poor delayed-type hypersensitivity response to specific islet antigens (eg. GAD) may be less likely to develop diabetes (Harrison et al., 1993). In addition, there is evidence from the non-obese diabetic (NOD) mouse model of spontaneous diabetes that GAD-responsive, IFN-γ producing T lymphocytes are an early feature of the disease process (Rapoport et al., 1993). The present study lends support to this hypothesis, in that the predominant circulating cytokine profile at diagnosis of Type 1 diabetes is T_H1, with little evidence of T_H2 cytokines.

High levels of cytokines in recently diagnosed Type 1 diabetes could be due to either the disease process, a non-specific effect of ongoing autoimmune disease, metabolic disturbances or non-specific clinical illness. Clinical illness may be associated with raised levels of cytokines in patients at diagnosis of diabetes but cannot explain the elevated levels in long-standing patients who were without clinical signs or symptoms of illness at the time of sampling. Alternatively, high levels of cytokines could be due to metabolic disturbances or a chronic autoimmune process. However, levels of cytokines did not correlate with patients' blood glucose levels and there was no relationship in patients with Type 1 diabetes between cytokine levels and the presence of antibodies to ICA or GAD. It is unlikely that the high levels of cytokines are genetically determined since we detected normal levels in long-standing Type 1 diabetic patients. It is likely therefore that the high levels of cytokines we detected are related to an immune process associated with Type 1 diabetes. This immune process is probably associated with the development of Type 1 diabetes since we demonstrated persistently elevated levels of activated T cells in

prediabetic twins but not in twins who did not develop Type 1 diabetes (Tun et al., 1994).

The results in this section confirm the finding of Cavallo et al. (1991) that levels of TNF-α are elevated in a proportion of newly-diagnosed patients with Type 1 diabetes, while levels of IL-4 and IL-6 are undetectable (Cavallo et al., 1991). In contrast, they found normal levels of IL-1α and IFN-γ in patients with Type 1 diabetes. This difference may be explained by differences in assay conditions or storage since cytokines are labile molecules with short half-lives; our serum samples were stored at -70°C and not -20°C as in that study. Elevated levels of TNF-α, but not IL-1α or IL-1ß, were found in a group of long-standing patients with hyperglycaemia by Mooradian et al, but the nature of the diabetes (Type 1 or Type 2) in these patients was not given (Mooradian et al., 1991). We were unable to detect IL-1β in serum, possibly due to the presence of cytokine inhibitors. Some of these receptors "binding protein", such as type II receptors, are naturally-occurring inhibitors for IL-1β but do not bind IL-1α (Scott et al., 1991).

High levels of TNF-α and IL-1 (macrophage-derived cytokine) in recently diagnosed patients with Type 1 diabetes indicate that cytokines may be involved in mediating β-cell damage. Previous studies have demonstrated that ß-cells are selectively and exquisitely sensitive to cytotoxic effects of IL-1α and IL-1ß (Palmer et al., 1989; Zawalich et al., 1989), whilst TNF-α and IFN-γ have a synergistic cytotoxic effect on islets (Pujol-Borrell et al., 1987). Thus, the macrophage could be an important effector of islet damage, since it is the main producer of these cytokines. Evidence from animal models of Type 1 diabetes supports such a role for macrophages (Hanenberg et al., 1989). In the BB rat, the appearance of macrophages in islets of Langerhans is the first event heralding the onset of insulitis (Walker et al., 1988). Manoeuvres designed to inhibit macrophage function, such as administration of silica, inhibit the development of diabetes in the BB rat (Oschilewski et al., 1985). In man, at diagnosis of diabetes, the islets of Langerhans show mononuclear cell infiltration, mainly by activated T cells and macrophages, and 40% of lymphocytes in this insulitis stain with antibody to IFN-γ (Foulis et al., 1991), a potent stimulator of macrophage activation (Pujol-Borrell et al., 1987). Thus, release of IFN-γ by activated T lymphocytes could lead to ß cell damage through inducing IL-1 and TNF release from macrophages (Hänninen et al., 1992).

Persistently high levels of cytokines in patients with established diabetes suggest that there is an unremitting, long-term immune process. C-peptide, an index of endogenous insulin secretion, and hence of intact β cells, did correlate with TNF-

α levels. By implication, therefore, the immune process which induces the production of increased TNF-α is still active as long as islet β cells remain. The mechanism by which autoimmune reactions are initiated against the β-cells and the stimulus for cytokine production in Type 1 diabetes are not known. Viral infection could initiate the immune process; viruses are capable of inducing IFN-γ (Trinchieri and Perussia, 1985) and have been shown to induce secretion of other cytokines such as TNF, IL-1β and IL-6 from human monocytes (Henke et al., 1992).

Levels of TNF-α, IL-2 and sIL-2 receptor were also raised in some patients with both long-standing Type 1 diabetes and Type 2 diabetes. These individuals are at risk of diabetic complications. Previous work in our laboratory has demonstrated high levels of circulating activated T lymphocytes in patients with symptoms and signs of diabetic neuropathy (Gilbey et al., 1988) and diabetic nephropathy (Bending et al., 1988). It is possible that complications of diabetes are associated with increased secretion of cytokines but further studies are required to address specifically this question.

In summary, this section shows that high levels of circulating IL-1α, TNF-α, IL-2, IFN-γ and sIL-2R are associated with the diagnosis of Type 1 diabetes. This supports the recent proposal that a balance in favour of T_H1 lymphocytes is a key element in the development of Type 1 diabetes. Whichever the stimulus leading to cytokine production in Type 1 diabetes, this immune system imbalance may precede the clinical onset of the disease. A more detailed analysis of the immune changes present in the prediabetic period will be presented in **chapter 6**.

CHAPTER 6

PROSPECTIVE STUDY OF CYTOKINE LEVELS IN IDENTICAL TWINS OF PATIENTS WITH TYPE 1 DIABETES

6.1 SUBJECTS

A consecutive series of non-diabetic identical co-twins of patients with Type 1 diabetes referred within 4 years of the diagnosis of the index twin and seen between June 1982 and January 1992 were recruited for this study. Monozygosity was established in all the twin pairs as previously described (Barnett *et al*., 1981) using a minimum of 22 blood group antigens. All the twins had oral glucose tolerance tests (75g or 1.75g/kg, whichever was the less) both initially and at intervals thereafter to confirm that they were not diabetic. On every occasion, each twin was shown to have a fasting whole blood glucose level less than 6.7 mmol/l (i.e. within the normal range). Twins were followed either because they had developed Type 1 diabetes, or remained non-diabetic for a minimum of 6 years after which period their risk of developing Type 1 diabetes is less than 2% (see section **4.1.4**). Twenty-eight identical co-twins fulfilled the criteria of entry into the study. The twins were ascertained within one year (n=22), 2 years (n=2), 3 years (n=1) and 4 years (n=3) of diagnosis of their index twin. Eight twins developed diabetes (prediabetic twins) (4 males; median age 13 years, range 10-20); they were tested on a median of 2 (range 2-8) occasions up to 5 years before diagnosis. Twenty twins (12 males; median age 19 years, range 7-53) remain non-diabetic (non-diabetic twins) (median follow up 11 years, range 6-19 years after diagnosis of their index twin). In the twins remaining non-diabetic for many years, multiple samples have been collected during follow-up. To facilitate the determination of changes in cytokine levels with time, samples from the non-diabetic twins were taken and measured on just two occasions, the first within 4 years of diagnosis of diabetes in the index twin, the second at least 5 years from diagnosis of the index twin.

As normal control subjects, 41 healthy individuals were studied and described in section **5.1**.

At the time of sampling, all twins and controls subjects were healthy and had no clinical signs or symptoms of intercurrent illness.

6.2 RESULTS

In this cohort of non-diabetic identical twins of patients with Type 1 diabetes, serum levels of IL-1α and TNF-α, IL-2 and IFN-γ and IL-4 and IL-10 were measured. Insufficient serum was available to measure levels of-IL-1β and IL-6.

The question being investigated in this section is whether abnormally elevated levels of cytokines (higher than the highest value in normal controls) is similar in prediabetic and non-diabetic twins. In order to avoid potential biases

caused by different numbers of measurements and different periods of follow-up for each twin, median levels of cytokines for each subject will be determined. If this is abnormally high, the subject is classified as having an abnormal levels of cytokines. The proportion of subjects with abnormal levels of cytokines in each group will be compared using the χ^2 test.

6.2.1 MACROPHAGE-DERIVED CYTOKINES

TNF-α. The number of twins who had abnormal median levels of TNF-α was higher in prediabetic than the non-diabetic twins group (χ^2=6, p<0.01) (**Figure and Table 6.1**).

Prediabetic and non-diabetic twins who had levels of TNF-α exceeding the highest normal value and whose serum was available were also tested for TNF-α bioactivity. Bioactive TNF-α was detected on 11/16 occasions (67%) in prediabetic twins (median 0.9 U/ml, range 0-17), 8 of whom had levels of TNF-α >1 u/ml and on 2/6 occasions (33%) in non-diabetic twins (median 0 U/ml, 0-2.3), only one of whom had TNF-α levels >1 U/ml.

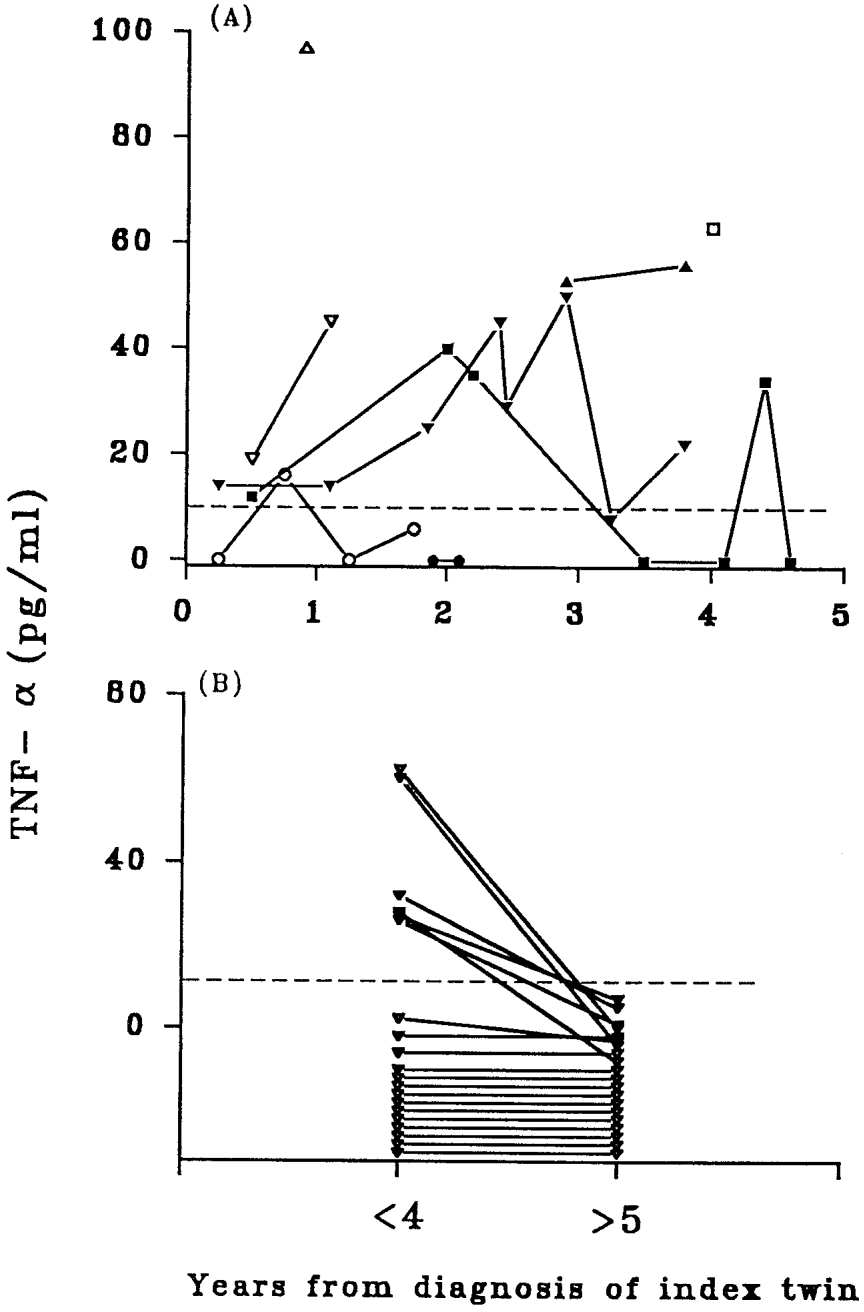


Figure 6.1. TNF-α levels in 8 prediabetic (A) and 20-non-diabetic (B) twins. The dashed line represents the upper limit of normal subjects. The number of twins who had abnormal median levels of TNF-α over time was higher in prediabetic than in non-diabetic twin group (χ^2=6, p<0.01)

IL-1α. The number of twins who had abnormal median levels of IL-1α was higher in prediabetic than the non-diabetic twins group (χ^2=13, p<0.0002) (**Figure. 6.2**) (**Table 6.1**).

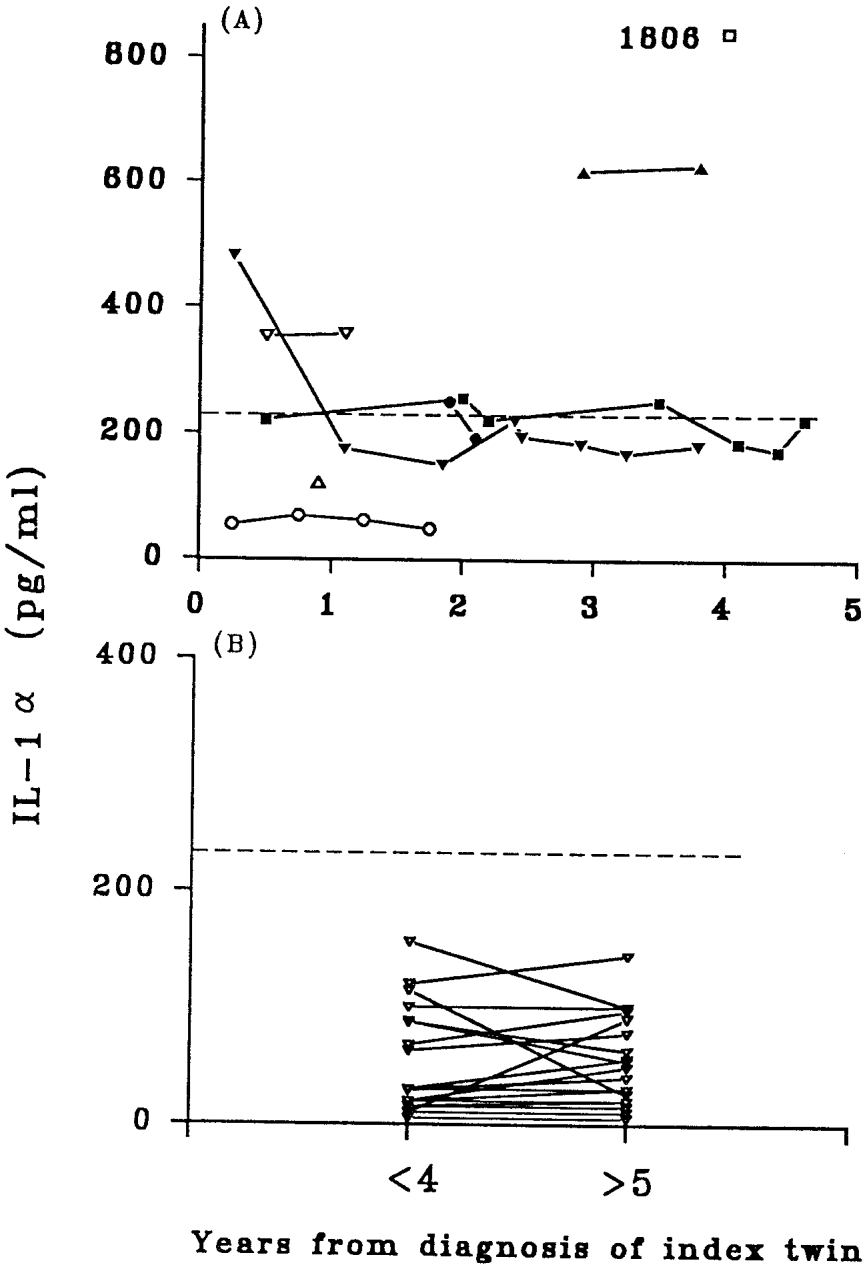


Figure 6.2. IL-1α levels in 8 prediabetic (A) and 20-non-diabetic (B) twins. The dashed line represents the upper limit of normal subjects. The number of twins who had abnormal median levels of IL-1α over time was higher in prediabetic than in non-diabetic twin group (χ^2=13, p<0.0002).

6.2.2 T_H1 PROFILE

IL-2. The number of twins who had abnormal median levels of IL-2 was not significantly different in prediabetic when compared with non-diabetic twins (χ^2=1.3, p<0.2) (**Figure. 6.3**) (**Table 6.1**).

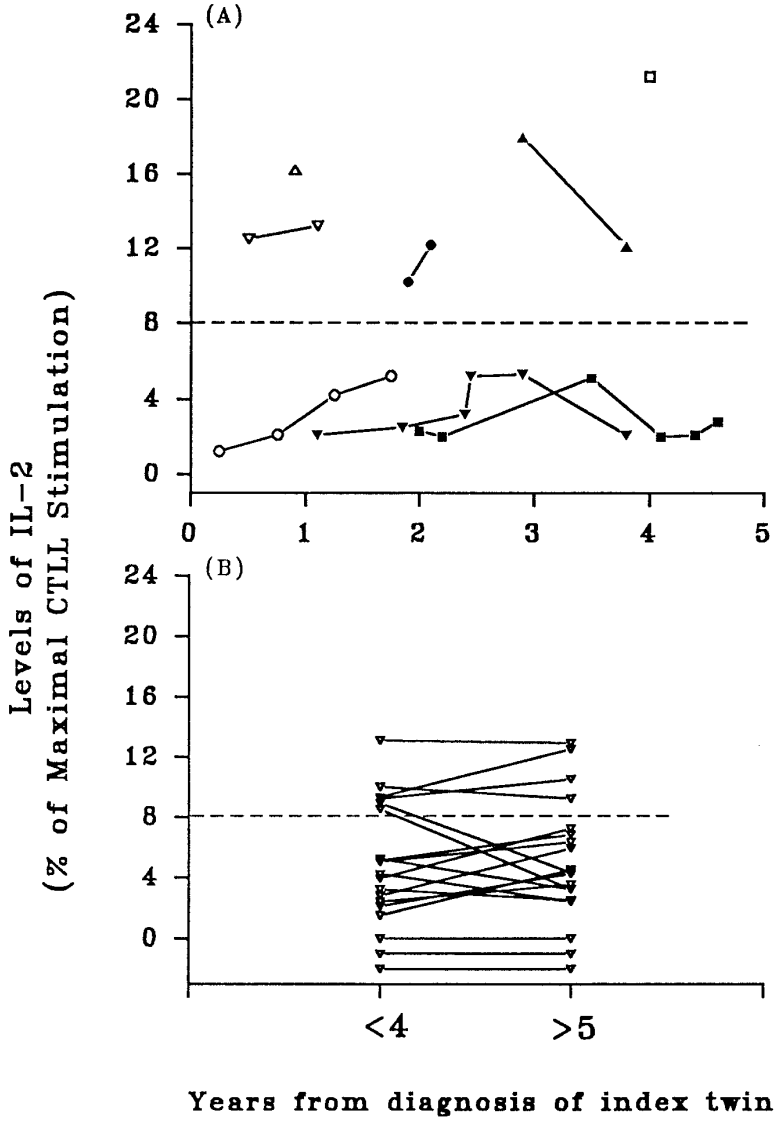


Figure 6.3. IL-2 levels in 8 prediabetic (A) and 20-non-diabetic (B) twins. The dashed line represents the upper limit of normal subjects. The number of twins who had abnormal median levels of IL-2 over time was not significantly different in prediabetic when compared with-non-diabetic twin group (χ^2=1.3, p<0.2).

IFN-γ. The number of twins who had abnormal median levels of IFN-γ was not significantly different in prediabetic when compared with non-diabetic twins (χ^2=3.5, p<0.06) (**Figure 6.4**) (**Table 6.1**).

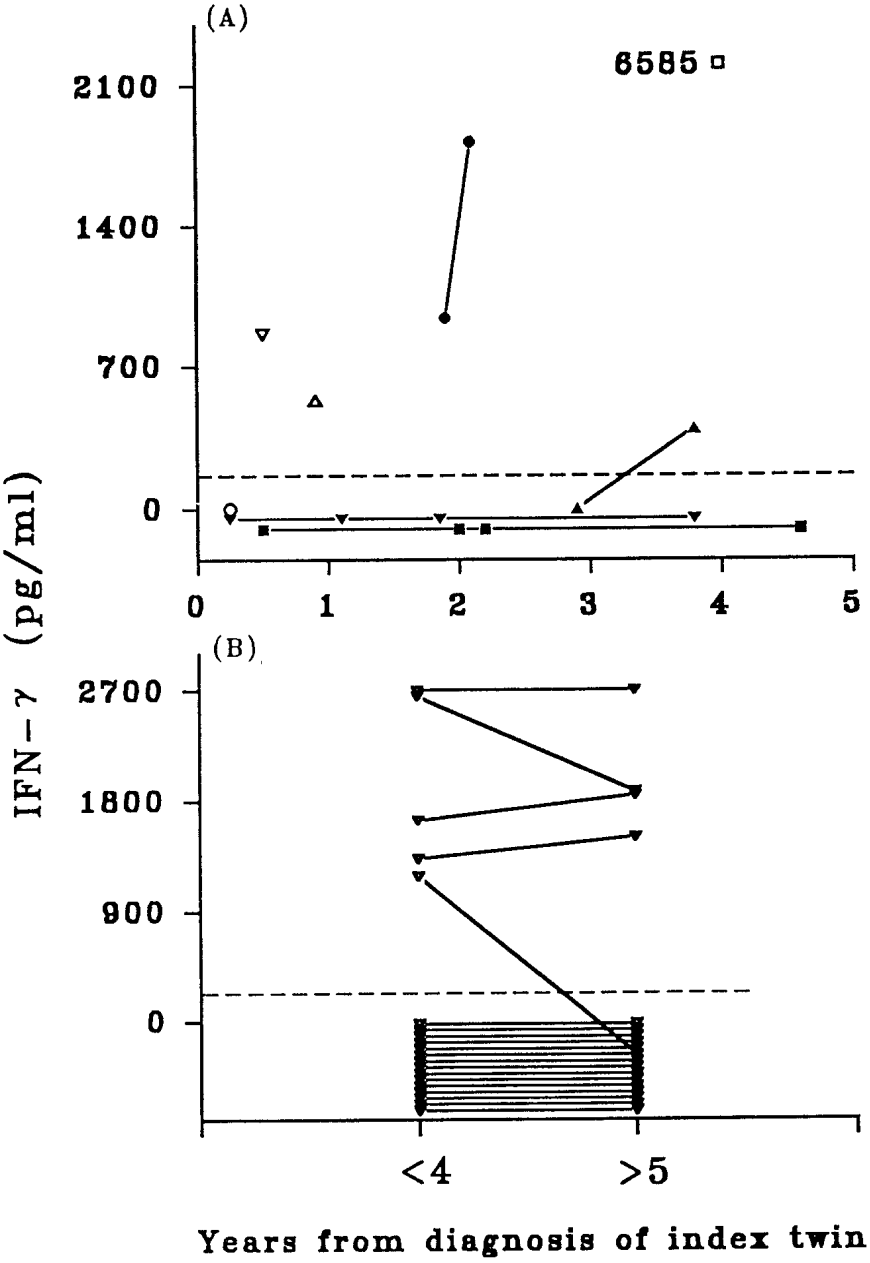


Figure 6.4. IFN-γ levels in 8 prediabetic (A) and 20-non-diabetic (B) twins. The dashed line represents the upper limit of normal subjects. The number of twins who had abnormal median levels of IFN-γ over time was not significantly different in prediabetic when compared with-non-diabetic twin group (χ^2=3.5, p<0.06).

6.2.3 T_H2 PROFILE

IL-4 and IL-10. The number of twins who had abnormal median levels of IL-4 and IL-10 was not significantly different in prediabetic when compared with non-diabetic twins (χ^2=0.03, p<0.9 for IL-4 and χ^2=0.43, p<0.5 for-IL-10) (**Figure 6.5 and Figure 6.6**) (**Table 6.1**).

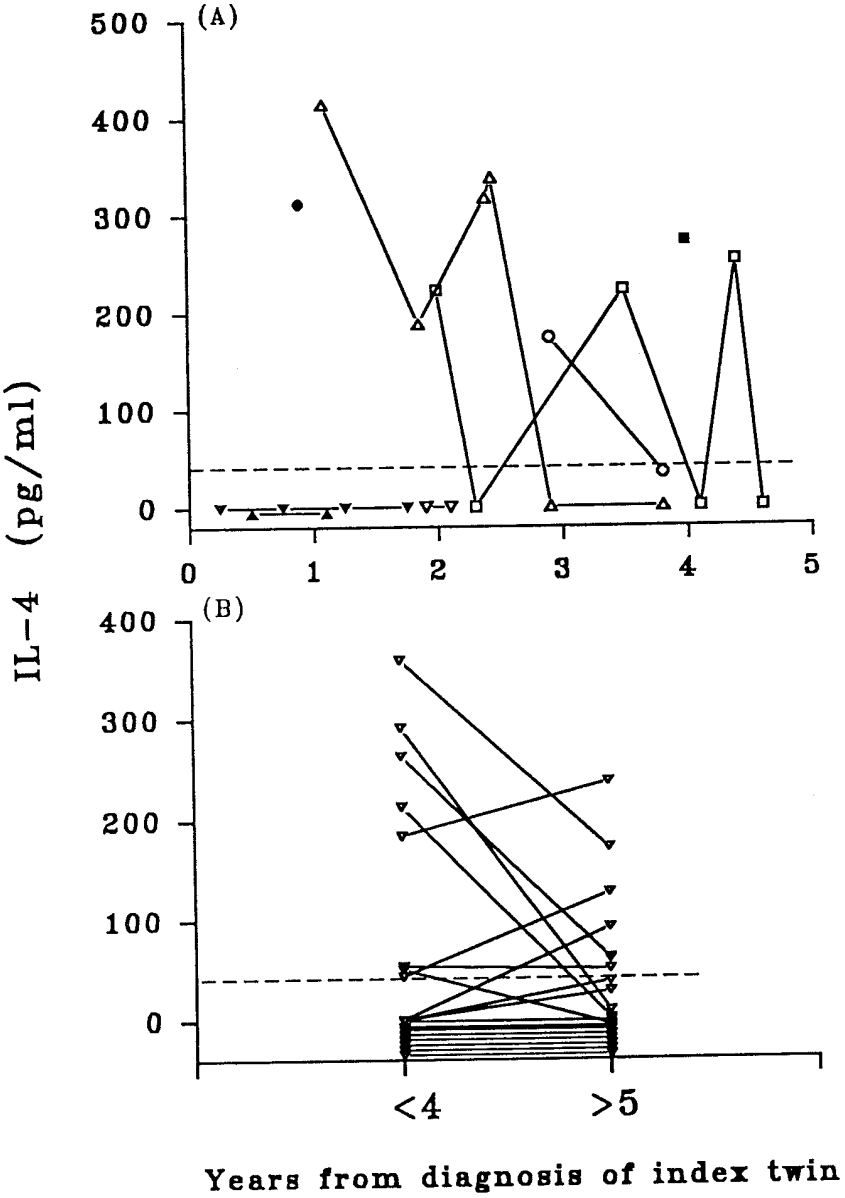


Figure 6.5. Levels of IL-4 in 8 prediabetic (A) and 20 non-diabetic (B) twins. The dashed line represents the upper limit of normal subjects. The number of twins who had abnormal median levels of IL-4 over time was not significantly different in prediabetic when compared with non-diabetic twin group (χ^2=0.03, p<0.9).

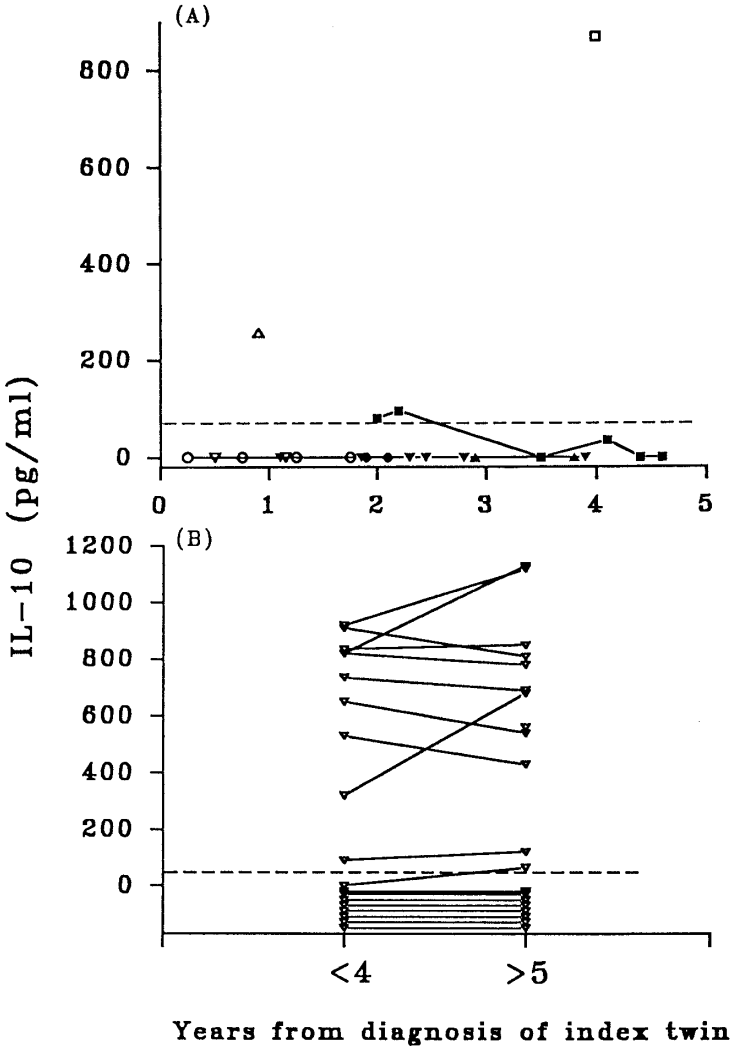


Figure 6.6. Levels of IL-10 in 8 prediabetic (A) and 20 non-diabetic (B) twins. The dashed line represents the upper limit of normal subjects. The number of twins who had abnormal median levels of IL-10 over time was not significantly different in prediabetic when compared with non-diabetic twin group (χ^2=0.43, p<0.5).

6.2.4 PREVALENCE OF T_H1 AND T_H2 CYTOKINE PROFILES IN IDENTICAL TWINS OF PATIENTS WITH TYPE 1 DIABETES

To examine whether a pattern of T_H1 or T_H2 dominance existed in prediabetic or non-diabetic twins, cytokine profiles were classified as T_H1 (elevated levels of IL-2 or IFN-γ) or T_H2 (elevated levels of IL-4 or IL-10). In prediabetic twins, 3/8 (37%) had a predominantly T_H1 and 2/8 (25%) T_H2. In the remainder, neither T helper subset was dominant. In twins remaining non-diabetic, 1/20 (5%) had a predominantly T_H1 profile and 8/20 (40%) T_H2, whilst in the remainder neither subset was dominant.

6.2.5 COMPARISON OF PREVALENCE OF T_H1 AND T_H2 CYTOKINE PROFILES IN IDENTICAL TWINS AND PATIENTS WITH TYPE 1 DIABETES

The number of individuals who had abnormal median levels of T_H1 (IL-2 and IFN-γ) and T_H2 (IL-4 and IL-10) cytokines were compared between recently diagnosed patients with Type 1 diabetes and groups of prediabetic and non-diabetic twins using χ^2 test.

T_H1 cytokines. The number of individuals who had abnormal median levels of T_H1 cytokines was significantly higher in patients with recently diagnosed Type 1 diabetes than in non-diabetic twins ($p<0.01$ for IL-2 and $p<0.03$ for IFN-γ) (**Table 6.1**). No difference in levels of T_H1 cytokines between recently diagnosed patients with Type 1 diabetes and prediabetic twins was observed.

T_H2 cytokine. The number of individuals who had abnormal median levels of T_H2 cytokines was significantly higher in both prediabetic and non-diabetic twins than in patients with recently diagnosed Type 1 diabetes ($p<0.0001$ and $p<0.001$ for IL-4 and $p<0.03$ and $p<0.0005$ for IL-10 respectively) (**Table 6.1**).

Table 6.1 Comparison of prevalence of elevated levels of macrophage-derived cytokines and T_H1 (IL-2 and IFN-γ) and T_H2 (IL-4 and IL-10) cytokine profiles in identical twins and patients with Type 1 diabetes.

				p values*		
Cytokines	**Prediabetic twins**	**Non-diabetic twins**	**Recently diagnosed Type 1 diabetics**	**Prediabetic twins v. recently diagnosed Type 1 diabetics**	**Non-diabetic twins v. recently diagnosed Type 1 diabetics**	**Prediabetic v. non-diabetic twins**
TNF-α (Mø)	7/8 (88%)	6/20 (30%)	23/31 (74%)	NS	0.005	0.01
IL-1α (Mø)	6/8 (75%)	0/20 (0%)	18/31 (58%)	NS	0.0001	0.0002
IL-2 (T_H1)	5/8 (63%)	6/20 (30%)	20/31 (64%)	NS	0.01	NS
IFN-γ (T_H1)	5/8 (63%)	5/20 (25%)	18/31 (58%)	NS	0.03	NS
IL-4 (T_H2)	5/8 (63%)	10/20 (50%)	3/31 (9%)	0.0001	0.001	NS
IL-10 (T_H2)	3/8 (38%)	11/20 (55%)	1/31 (3%)	0.03	0.0005	NS

*Comparisons were made between recently diagnosed patients with Type 1 diabetes and groups of prediabetic and non-diabetic twins using the χ^2 test.

6.2.6 ASSOCIATION BETWEEN LEVELS OF CYTOKINES, BLOOD GLUCOSE LEVELS AND AUTOANTIBODIES IN IDENTICAL TWINS

The mean (± SD) glucose level of all the blood samples obtained from prediabetic (n=22) and non-diabetic twins (n=35) were 4.3 ± 0.7 mmol/l (range 3-5.3 mmol/l) and 4.7± 2 mmol/l (range 2.8-5.1 mmol/l) respectively. No correlation was found between levels of cytokines and blood glucose levels in either prediabetic or non-diabetic twins.

Islet cell antibodies (ICA) and GAD antibodies were detected in all but one of the 8 prediabetic twins in the first blood sample available for cytokine studies. All but two of the 20 twins who remain non-diabetic were negative for ICA and all but one were negative for GAD antibodies in the first blood sample available for cytokine studies. No correlation was found between levels of cytokines and the presence of ICA or anti-GAD antibodies in either prediabetic and non-diabetic twins.

In non-diabetic twins, levels of IL-4 were correlated with IL-10 ($r=0.47$, $p<0.002$) while levels of IL-2 were correlated with IFN-γ in both prediabetic and non-diabetic twins ($r=0.75$ and $r=0.65$, $p<0.002$ and 0.0001 respectively).

6.3 COMMENT

Levels of macrophage-derived cytokines (IL-1α and TNF-α) were elevated more frequently in prediabetic twins who developed diabetes than in non-diabetic twins who did not. In contrast, cytokine profiles classified as T_H1 (elevated levels of IL-2 or IFN-γ) or T_H2 (elevated levels of IL-4 and IL-10) existed in both prediabetic or non-diabetic twins without dominance of either T_H1 or T_H2.

Elevated levels of TNF-α and IL-1α in identical twins at risk of developing Type 1 diabetes indicate that the predominant cytokines during the prediabetic period are macrophage-derived (TNF-α and IL-1α). These results are consistent with the observation of a macrophage infiltration as the first event heralding the onset of insulitis in the NOD mouse and BB rat models of diabetes as discussed previously (vide **5.3**). Thus, elevated levels of circulating IL-1α and TNF-α in prediabetic twins suggest that the macrophage could be an important effector, and macrophage-derived cytokines an important marker of islet damage.

The presence of elevated levels of IL-1α and TNF-α in prediabetic twins long before there is hyperglycaemia indicates that an elevated levels of these cytokines is unlikely to be an epiphenomenon secondary to metabolic disturbance or clinical illness. In addition, there was no relationship between cytokine levels and the presence of antibodies to ICA or GAD. It is unlikely that the high levels of cytokines in prediabetic twins are genetically determined since normal levels were detected in the-non-diabetic twins of diabetic patients. It is likely therefore that the high levels of cytokines detected are related to an immune process associated with the development of Type 1 diabetes since persistently elevated levels of both TNF-α and IL-1α in prediabetic twins but not in twins who did not develop Type 1 diabetes were demonstrated in the present study.

As in recently diagnosed patients with Type 1 diabetes, the mechanism by which autoimmune reactions are initiated against the β-cells and the stimulus for cytokine production in prediabetic twins are not known but may involve viral infection (vide **5.3**).

Although some prediabetic twins had primarily a T_H1 profile of circulating cytokines, there was no clear pattern of T_H1 or T_H2 profile in twins destined to become diabetic, or in those remaining non-diabetic and unlikely to develop the disease. However, levels of cytokines typical of a T_H2 profile were higher in non-diabetic twins than in patients with recently diagnosed Type 1 diabetes. This finding supports a possible role for T_H2 cytokines (IL-4 and IL-10) in preventing the disease processes by down-regulating autoimmune responsiveness. Recent evidence

supports the latter concept and it has been shown that IL-4 (Rapoport *et al*., 1993; Fowell and Mason, 1993) and IL-10 (Pennline, *et al*., 1994) prevent the onset of diabetes in NOD mice suggesting the role of T_H2 cytokines in down-regulating the IFN-γ-producing T_H1 subset, an important mediator of islet β-cell destruction (Rabinovitch, 1994). This is supported by evidence that the two functional subsets are mutually antagonistic. For example, IL-10 suppresses synthesis of T_H1 helper cytokines such as IL-2 and IFN-γ (Moore *et a*l., 1990; Vieira *et al*., 1991). It has also been shown that IL-4 suppresses LPS-induced stimulation of IL-1, TNF-α and-IL-6 synthesis by human monocytes (Cheung *et al*., 1990). These observations are in keeping with the finding in the present study that high levels of IL-4 and IL-10 in-non-diabetic twins are associated with low levels of TNF-α and IL-1α and that levels of T_H1 cytokines are lower than T_H2 cytokines.

In conclusion, these data show that macrophage-derived cytokines (IL-1α and TNF-α) are consistently raised throughout the prediabetic period. This supports the hypothesis that macrophages could be important effectors of islet damage. Analysis of T_H1 or T_H2 profiles of twin groups did not reveal a clear difference in the pattern obtained in the prediabetic twins compared with that in the twins remaining non-diabetic. The increased levels of T_H2 cytokines in the non-diabetic twins but not in patients with diabetes may give support to the role of T_H2 cells in preventing the disease.

CHAPTER 7

STUDIES ON CYTOKINE LEVELS AND THEIR RELATIONSHIP TO AUTOANTIBODIES AND HLA ALLELES IN DIABETIC CHILDREN AND THEIR FIRST DEGREE RELATIVES

7.1 SUBJECTS

Twenty-nine families, all with an index case of Type 1 diabetes attending the District Diabetes Centre, Farnborough Hospital, Kent, were studied. All families with children attending the Diabetes Centre were approached and all those willing to participate were recruited. As far as possible, blood samples were obtained from all siblings and both parents. The composition of the families studied is shown in **Table 7.1**.

There were a total of 29 children with Type 1 diabetes (14 males, median age 13 years, range 2-15) with a mean duration of diabetes of 3.1 ± 1.5 years. Duration of diabetes ranged from 3 months to 5 years, and 3 diabetic children were studied within 1 year of diagnosis. Type 1 diabetes was defined in accordance with the guidelines of the National Diabetes Data Group. At the time of sampling, the mean random blood glucose level amongst the diabetic children was 11.9 ± 5.5 mmol/l and the mean level of haemoglobulin A_1C was 10.1 ± 1.8%. In addition, we studied 51 parents, (25 males, median age 42 years, 30-46) and 34 siblings (15 males, median age 11 years, 3-20), none of whom had diabetes (mean ± SD level of haemoglobulin A_1C 6.8% ± 0.7 in the siblings and 6.7% ± 0.7 in the parents).

As normal control subjects, we studied 41 healthy individuals as described in section 5.1.

Table 7.1 Composition of families studied

Number of Families	Mother	Father	Diabetic	Sib 1	Sib 2	Sib 3
8	_	_	_	_	_	
12	_	_	_	_		
2	_	_	_			
1	_		_	_	_	_
1	_		_	_	_	
1	_		_	_		
3	_		_			
1		_	_			

7.2 RESULTS

Serum levels of TNF-α, IL-1α, IFN-γ and sIL-2R in 29 children with Type 1 diabetes and their first degree relatives (parents and siblings) and their relationship to HLA type and autoantibodies, are described in this section.

7.2.1 CYTOKINE AND sIL-2R LEVELS.

TNF-α. Levels of TNF-α were significantly increased not only in children with Type 1 diabetes but also in their non-diabetic siblings and parents when compared with the appropriate control group ($p<0.02$, for all) (**Figure 7.1**). Levels of TNF-α exceeding the highest normal value (>9 pg/ml) were found in 13/29 (45%) diabetic children, 6/28 (21%) of their siblings and 6/33 (18%) of their parents.

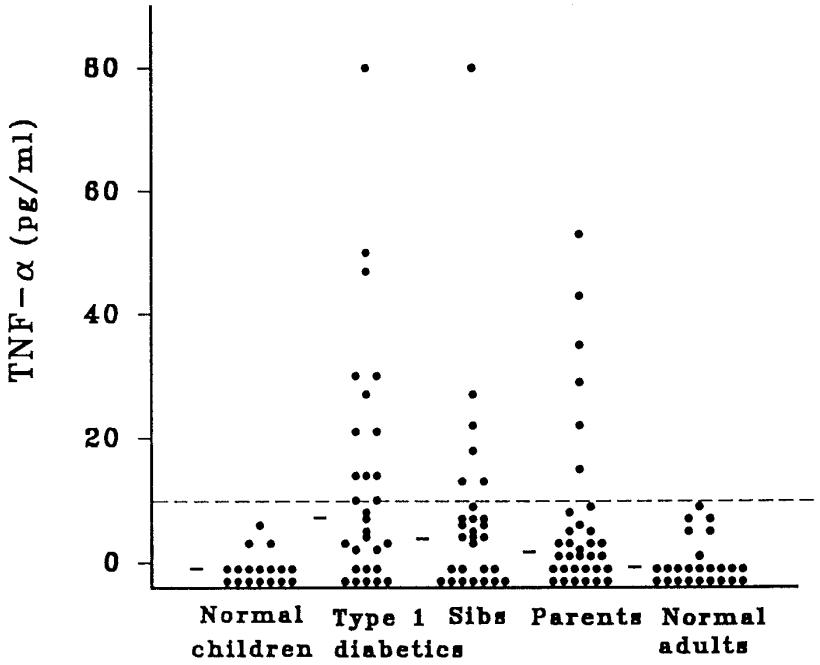


Figure 7.1. TNF-α values in the serum of children with Type 1 diabetes, in their parents and siblings and controls. The dashed line represents the upper limit of normal controls. Levels of TNF-α were significantly higher in children with Type 1 diabetes, in their siblings and parents ($p<0.02$ for all) when compared with normal controls. Horizontal bars represent median values.

IL-1α. A similar pattern was seen for IL-1α. However, although the levels were increased in the diabetic children, their siblings and parents, these elevations did not reach statistical significance (**Figure 7.2**). Levels of IL-1α exceeding the highest normal value (>192 pg/ml) were found in 7/29 (24%) diabetic children, 8/33 (24%) of their siblings and 9/32 (28%) of their parents.

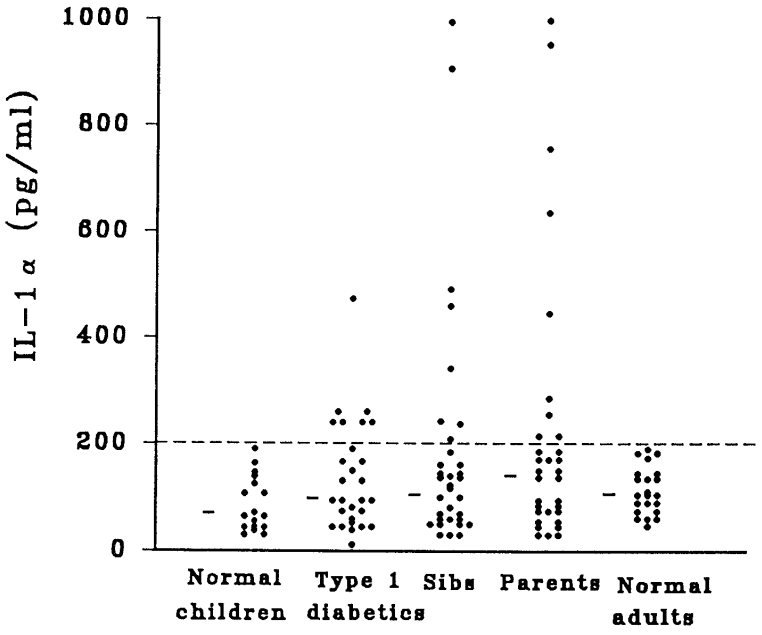


Figure 7.2. IL-1α values in the serum of children with Type 1 diabetes, in their parents and siblings and controls. The dashed line represents the upper limit of normal controls. Levels of IL-1 were not significantly higher in children with Type 1 diabetes, in their siblings and parents when compared with normal controls. Horizontal bars represent median vales.

IFN-γ. A similar pattern was also seen for IFN-γ. There was no significant difference in the levels of IFN-γ between diabetic children, their siblings and parents and normal controls (**Figure 7.3**). Levels of IFN-γ exceeding the highest normal value (>250 pg/ml) were found in 8/27 (30%) diabetic children, 8/26 (31%) of their siblings and 7/26 (27%) of their parents.

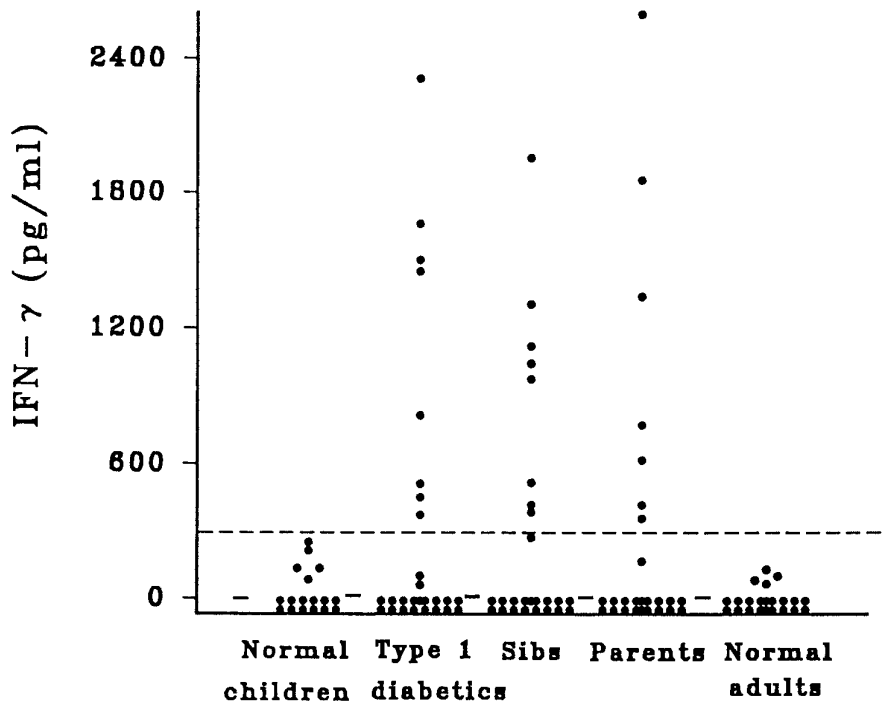


Figure 7.3. IFN-γ values in the serum of children with Type 1 diabetes, in their parents and siblings and controls. The dashed line represents the upper limit of normal controls. Levels of IFN-γ were not significantly higher in children with Type 1 diabetes, in their siblings and parents when compared with normal controls. Horizontal bars represent median values.

sIL-2R. Levels of sIL-2R were significantly increased not only in diabetic children but also in their siblings and parents when compared with appropriate normal controls ($p<0.001$, for all) (**Figure 7.4**). Levels of sIL-2R exceeding the highest normal value (>410 U/ml) were found in 9/22 (40%) diabetic children, 11/20 (55%) of their siblings and 20/50 (40%) of their parents.

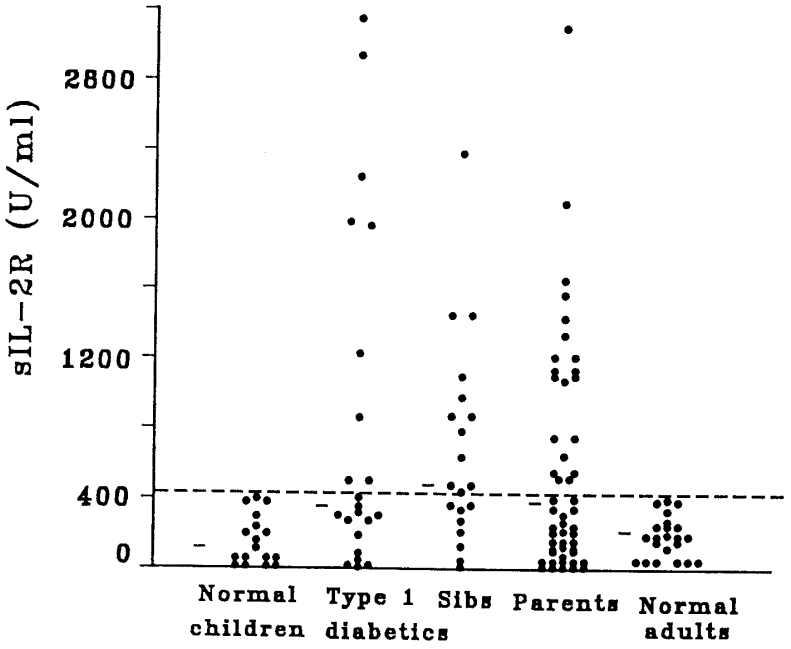


Figure 7.4. sIL-2R values in the serum of children with Type 1 diabetes, in their parents and siblings and controls. The dashed line represents the upper limit of normal controls. Levels of sIL-2R were significantly higher in children with Type 1 diabetes, in their siblings and parents ($p<0.001$ for all) when compared with normal controls. Horizontal bars represent median values.

7.2.2 ASSOCIATION BETWEEN LEVELS OF CYTOKINES AND ISLET CELL ANTIBODIES OR OTHER AUTOANTIBODIES.

Islet cell antibodies (ICA) were detected in 18 diabetic children (62%), 4 siblings (12%) and 2 parents (4%). Diabetic children with ICA had higher levels of IFN-γ compared with their ICA-negative counterparts (median 100 pg/ml, range 0-2310 versus 0 pg/ml, 0-615; $p<0.05$). In addition, in patients with ICA, levels of IFN-γ were higher at times near to diagnosis than more distant from diagnosis. Levels of TNF-α, IL-1α and sIL-2R were similar in siblings, parents or diabetic children irrespective of the presence or absence of ICA.

Autoantibodies other than ICA (such as GPC, TMA, TGA, ANA, SMA, ARA, AMA) were detected in 6 diabetic children (19%), 8 siblings (24%) and 15 parents (29%) (**Table 7.2**). In 1 diabetic child, 4 siblings and 6 parents more than

one autoantibody was present, usually as a combination of GPC and TMA which accounted for 6 of the cases of multiple autoantibodies. There were no differences in levels of cytokines studied when diabetic children or their siblings and parents were divided according to the presence or absence of 1 or more of the non-ICA autoantibodies.

No correlation was found between TNF-α, IL-1α and IFN-γ levels in diabetic children, their siblings or their parents.

Table 7.2 Prevalence (%) of organ-specific (excluding islet cell antibody) and non organ-specific autoantibodies in children with Type 1 diabetes, their siblings and parents and controls.

Subjects	**GPC n (%)**	**TMA n (%)**	**TGA n (%)**	**ANA n (%)**	**SMA n (%)**	**ARA n (%)**	**AMA n (%)**	**≥1 of all types n (%)**
Patients with Type 1 diabetes	6 (19)	3 (10)	1 (3)	neg	neg	neg	neg	6 (19)
Non-diabetic siblings	3 (9)	5 (15)	Neg	1 (3)	1 (3)	1 (3)	2 (6)	8 (24)
Non-diabetic parents	8 (15)	11 (20)	2 (4)	1 (2)	1 (2)	2 (4)	neg	15 (29)
Healthy children	neg	neg	Neg	neg	neg	neg	neg	0 (0)
Healthy adults	1 (4)	neg	Neg	neg	neg	neg	neg	1 (4)

Abbreviations: GPC (gastric parietal cell antibody); TMA (thyroid microsomal antibody); TGA (thyroglobulin antibody); ANA (anti-nuclear antibody); SMA (smooth muscle antibody); ARA (anti-reticulin antibody); AMA (anti-mitochondrial antibody).

7.2.3 ASSOCIATION BETWEEN LEVELS OF CYTOKINES AND HLA CLASS II ALLELES.

In previous studies it has been shown that approximately 90% of children with Type 1 diabetes possess at least one DR3 or DR4 allele, and it is likely that an influence of class II loci on immune function in this disease could operate through these two genes. For this reason, and because of the small numbers in each study group, individuals were assigned to the following 4 groups: These groups are :(1) possession of homozygous DR3/3 or heterozygous DR3/X; (2) heterozygous DR3/4; (3) homozygous DR4/4 or heterozygous DR4/X; and (4) DRX/X (where X is not DR3 or DR4). Amongst the diabetic children, 7 (23%) were DR3/3 or DR3/X, 13 (42%) were DR3/4, 9 (29%) were DR4/4 or DR4/X and 2 (6%) were DRX/X so that 94% of the diabetic children possessed at least one of the DR3 or DR4 alleles. Amongst the siblings, 12 (35%) were DR3/3 or DR3/X, 6 (18%) were DR3/4, 6 (18%) were DR4/4 or DR4/x and 10 (29%) were DRX/X. The frequency of certain haplotypes was different in the diabetic children compared with the siblings. A significantly higher proportion of the diabetic children were DR3/4 heterozygous (Fisher's exact probability $p=0.029$), whilst significantly more of the siblings possessed alleles other than DR3 or DR4 (DRX/X; Fisher's exact probability $p=0.018$). Three of the parents were not HLA typed. Of the remainder, 18 (35%) were DR3/3 homozygous or DR3/X, 12 (23%) were DR3/4 heterozygous, 12 (23%) were DR4/4 or DR4/X and 10 (18%) were DRX/X.

No differences were seen in levels of cytokines between any of these groups in diabetic children, siblings or parents.

7.2.4 ASSOCIATION BETWEEN LEVELS OF CYTOKINES, METABOLIC CONTROL AND DURATION OF DIABETES.

Amongst the diabetic children, there was no relationship between the increased levels of cytokines and random blood glucose, haemoglobulin A_1C, or duration of diabetes. To further examine the influence of metabolic control on cytokine release by lymphocytes, patients with Type 1 diabetes were divided into those with haemoglobulin A_1C levels <10.0 and those >10.0. Again, none of the cytokine and sIL-2R levels were different in the two groups of diabetic children compared with controls and with each other.

7.3 COMMENT

The study reported in this section shows that circulating levels of TNF-α and sIL-2R are present in non-diabetic first degree relatives (i.e. siblings and parents) of children with Type 1 diabetes, as well as in the diabetic children themselves.

The observation of elevated levels of TNF-α and sIL-2R in this different study population confirms the observation detailed in section 5.2 and indicates the activity of macrophages and T cells to be closely linked to Type 1 diabetes.

The finding that elevated levels of TNF-α and sIL-2R are also present in siblings and parents of patients with Type 1 diabetes, though at lower levels is intriguing. Siblings of children with Type 1 diabetes, despite often sharing environmental and genetic risk of diabetes with diabetic members of their family, have a relatively low risk of developing the disease themselves (Allen *et al*., 1991). Thus, the elevated cytokine levels observed in siblings are unlikely to signify impending diabetes at least in the majority of the individuals studied. One possible explanation for the increased levels of TNF-α and sIL-2R in the first degree relatives of diabetic children is that the immune system is hyperactive in these individuals. Such an overactive immune system has been incriminated as a possible predisposing factor to autoimmune diseases in individuals with the haplotype A1 B8 DR3, who have also been reported to have impaired Fc receptor function (Lawley et al., 1981) and defective suppressor-cell function (Jaworski *et al*., 1983). The concept that genetic factors are responsible for an overactive immune system - exemplified by elevated levels of cytokines - does not appear to be sustained by the present data. Thus, no association was noted between elevated levels of cytokines and sIL-2R and any specific HLA class II allele (HLA-DR3/4), neither in diabetic children nor their relatives, suggesting that the DR3/4 alleles do not exercise a univocal influence on determining high cytokine levels. It is possible that hypersecretion of cytokines and sIL-2R is associated with other alleles not investigated in the present work. In relation to the present study it is of interest that the genes encoding the cytokines TNF-α and TNF-β are located on the short arm of chromosome 6, between the HLA B and HLA DR loci (Campbell and Trowsdale, 1993). Furthermore an allelic polymorphism within the TNF-α promotor region which is strongly associated with the HLA A1, B8 and DR3 alleles has been reported (Wilson et al., 1993) and it has been suggested that such polymorphisms may be important in understanding Type 1 diabetes (Mølvig et al., 1990) and other autoimmune diseases (Fugger et al., 1989).

Increased levels of TNF-α and sIL-2R in diabetic children could be due to either the disease process, a non-specific effect of ongoing autoimmune disease,

metabolic disturbances or non-specific clinical illness. Clinical illness could be responsible for raised levels of TNF-α and sIL-2R in children with diabetes but cannot explain this elevation in their siblings and parents who were without clinical signs or symptoms of illness at the time of sampling. High levels of cytokines could also be due to metabolic disturbances or a chronic autoimmune process. However levels of cytokines did not correlate with blood glucose levels, haemoglobulin A_1C or presence of ICA or other autoantibodies.

Levels of other cytokines such as IL-1α and IFN-γ tended to be higher in diabetic children than in normal controls but the difference did not reach statistical significance. At first sight, these findings are in contrast with previous results in this thesis showing that levels of IL-1α and IFN-γ are elevated in patients with Type 1 diabetes at diagnosis (vide 5.2). The apparent difference could be explained by the fact that the mean duration of diabetes in the present study was more than 3 years. It has been suggested that immune abnormalities begin to disappear 6 months after diagnosis, and are usually normal in patients with long-term disease (Drell and Notkins, 1987; Gilbey et al., 1988; Bending et al., 1988). The results observed are in agreement with previous findings by Mooradian et al., (1991) who showed that levels of IL-1α and IL-1ß in a group of long-standing patients with hyperglycaemia were not different when compared with normal subjects, although the nature of the diabetes (Type 1 or Type 2) in those patients was not given. In contrast with the results presented here, Tova et al. (1984) found increased levels of IFN-γ in newly diagnosed children with Type 1 diabetes. This difference could be explained by the duration of the disease in the patients studied. Interestingly, in the present study, in patients with ICA, levels of IFN-γ were higher at times near to diagnosis than more distant from diagnosis, suggesting that levels of IFN-γ are associated with the presence of ICA, levels of which decline with time.

In summary, increased circulating levels of TNF-α and sIL-2R are present in diabetic children and give support to the hypothesis that activation of macrophages and T cells is linked to Type 1 diabetes. Elevated levels of cytokines and sIL-2R were also present in first degree relatives of children with Type 1 diabetes. Some of these abnormalities may be influenced by genes of the HLA system, and could represent one component of the susceptibility conferred by inheritance of particular alleles.

CHAPTER 8

STUDIES OF CYTOKINE SECRETION BY PERIPHERAL BLOOD MONONUCLEAR CELLS IN DIABETIC CHILDREN AND THEIR FIRST DEGREE RELATIVES

8.1 SUBJECTS

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised blood obtained from family members of patients with Type 1 diabetes and tested for cytokine production in vitro. The study groups comprised 19 children with Type 1 diabetes (12 males, median age 10, range 2-13 years), 23 parents (11 males, median age 35, 30-46 years) and 14 siblings (10 males, median age 10, 3-15 years). In addition, 39 healthy individuals comprising 19 adults (12 males, mean age 25 ± 9.1 years) and 20 children (11 males, mean age 12.1 ± 5.1 years) were studied. The individuals studied in this section were chosen from the subjects described in section 7.1. Selection being based on the availability of adequate amount of fresh, heparinised blood.

8.2 RESULTS

Cytokine secretion induced from PBMC from first degree relatives (parents and siblings from 19 families) of patients with Type 1 diabetes was measured. Lipopolysaccharride (LPS) was used to induce IL-1α and TNF-α from macrophages while phytohaemagglutinin (PHA) was used to induce IL-2 and IFN-γ from T cells.

8.2.1 TNF-α AND IL-1α SECRETION

TNF-α secretion: Levels of spontaneous TNF-α secretion were not different in diabetic children, their siblings and parents when compared with normal controls. LPS-stimulated TNF-α secretion was elevated in diabetic children ($p < 0.02$), their siblings and parents ($p < 0.0001$ and 0.001 respectively) when compared with normal controls (**Table 8.1**).

Table 8.1. Levels of spontaneous and LPS-stimulated secretion of TNF-α by PBMC in diabetic children, their siblings and parents.

Groups	no.	(spontaneous) TNF-α (pg/ml) Median Range	(with LPS) TNF-α (pg/ml) Median Range
Normal Controls	39	47 (0-52)	372 (138-701)
Diabetic Children	19	22 (0-544)†	2279 (54-3761)*
Siblings	14	57 (0-734)†	1385 (165-2134)*
Parents	23	80 (9-642)†	2205 (605-3483)*

†no significant difference compared with normal controls using Wilcoxon's rank sum test.
*$p<0.01$ compared with normal controls.

IL-1α secretion: Levels of spontaneous IL-1α secretion by mononuclear cells were increased in diabetic children, their siblings and parents when compared with normal controls ($p<0.03$, 0.001 and 0.01 respectively). A similar pattern of increase was also observed after LPS-stimulation in all groups studied when compared with normal controls ($p<0.003$, 0.003, 0.02 respectively) (**Table 8.2**).

Table 8.2 Levels of spontaneous and LPS-stimulated secretion of IL-1α by PBMC and after LPS stimulation in diabetic children, their siblings and parents.

Groups	**no.**	**(spontaneous) IL-1α (pg/ml) Median Range**	**(with LPS) IL-1α (pg/ml) Median Range**
Normal controls	39	0 (0-12)	82 (23-509)
Diabetic Children	19	0 (0-282)*	195 (24-741)*
Siblings	14	23 (0-283)*	341 (110-915)*
Parents	23	0 (0-190)*	295 (0-626)*

*$p<0.01$ compared with normal controls using Wilcoxon's rank sum test.

8.2.2 IL-2 AND IFN-γ SECRETION

IL-2 secretion: Levels of PHA-induced secretion of IL-2 (calculated as the percentage of maximal cytotoxic T-lymphocyte line (CTLL) stimulation) by PBMC were not different between diabetic children (median 35%, range 20-59), their siblings (33%, 9-38) and parents (27%, 7-45) and normal controls (32%, 11-64) (**Table 8.3**).

Table 8.3 Levels of spontaneous and PHA-stimulated secretion of IL-2 by PBMC in diabetic children, their siblings and parents.

Groups	no.	(spontaneous) IL-2 (% of CTLL stimulation) Median Range	(with PHA) IL-2 (% of CTLL stimulation) Median Range
Normal controls	39	0 (0-0)	32 (11-64)
Diabetic Children	19	0 (0-0)	35 (20-59)
Siblings	14	0 (0-0)	34 (9-38)
Parents	23	0 (0-0)	28 (9-45)

IFN-γ secretion: A similar pattern was obtained for IFN-γ. Levels of PHA-induced IFN-γ secretion by PBMC were not different between diabetic children (median 74 pg/ml, range 0-1200), their siblings (median 73 pg/ml, 0-1500) and parents (median 0 pg/ml, 0-650) when compared with normal controls (median 0 pg/ml, 0-360) (**Table 8.4**). Spontaneous secretion of both IL-2 and IFN-γ by PBMC was not detected in any group studied.

Table 8.4 Levels of spontaneous and PHA stimulated secretion of IFN-γ by PBMC in diabetic children, their siblings and parents.

Groups	no.	(spontaneous) IFN-γ (pg/ml) Median Range	(with PHA) IFN-γ (pg/ml) Median Range
Normal Controls	39	0 (0-0)	0 (0-246)
Diabetic Children	19	0 (0-0)	51 (0-1200)
Siblings	14	0 (0-0)	71 (0-950)
Parents	23	0 (0-0)	0 (0-650)

8.2.3 ASSOCIATION BETWEEN LEVELS OF CYTOKINE SECRETION AND ICA OR OTHER AUTOANTIBODIES.

There were no differences in the levels of cytokine secretion when diabetic children or their siblings and parents were divided according to the presence or absence of ICA, or of 1 or more of the non-pancreatic autoantibodies.

8.2.4 ASSOCIATION BETWEEN LEVELS OF CYTOKINE SECRETION, METABOLIC CONTROL, DURATION OF DIABETES AND HLA CLASS II ALLELES.

No correlation was found between levels of IL-1α, TNF-α, IL-2 and IFN-γ secretion and random blood glucose, HbA_1C, or duration of diabetes. In addition, no association between cytokine secretion profiles and HLA class II alleles was observed.

8.3 COMMENT

The results in this section demonstrate that LPS-stimulated IL-1α and TNF-α secretion by PBMC in vitro is significantly increased in diabetic children and their siblings and parents when compared with normal controls. The spontaneous secretion of IL-1α was also increased in diabetic children and their siblings and parents compared to normal controls. In contrast, levels of T-cell cytokines, IL-2 and IFN-γ, released after PHA stimulation were similar in diabetic children, their siblings and parents, and in normal controls.

The hypersecretion of TNF-α is in agreement with the studies in an animal model of diabetes of Rothe et al. who found that following activation by LPS, peritoneal macrophages from diabetes-prone BB rats secreted strikingly higher amounts of TNF-α than those found in diabetes-resistant or normal Wistar rats (Rothe et al., 1990). This contrasts with an earlier study in the NOD mouse by Satoh et al. who found that the induction of TNF-α in vitro after LPS stimulation was lower compared with normal animals (Satoh et al., 1989). Subsequent studies of TNF-α production by monocytes in patients with Type 1 diabetes have been reported but none have examined IL-1α. Mølviq et al. (1990), Mooradian et al. (1991) and Ohno et al. (1993) reported no alteration in TNF-α secretion.

Hypersecretion of TNF-α and IL-1α by PBMC of patients with Type 1 diabetes in the present study again indicates that the macrophage could be an important effector of islet damage since it is the main producer of TNF-α and IL-1α. TNF-α, in conjunction with IL-1 and IFN-γ, shows direct toxic effects on islet cells in vitro (Pukel *et al*., 1988). In addition, TNF-α and IL-1α are proinflammatory cytokines, the overproduction of which may favour chronic local inflammation such as pancreatic insulitis and thereby promote islet autoimmunity (Rothe et al., 1990).

The hypersecretion of IL-1α and TNF-α in siblings and parents is unlikely to represent imminent diabetes but it may represent hyperactivity of the immune system which is reported to be linked to particular HLA class II alleles (HLA-DR3/4) as discussed (vide 7.3). Absence of an association between hypersecretion of cytokines and HLA-DR3 or DR4 in both diabetic children and their relatives may indicate that other alleles not investigated in the present work could be linked to cytokine secretion as discussed in previous chapter (vide 8.3).

It is unlikely that the hypersecretion of TNF-α and IL-1α in patients with Type 1 diabetes observed in this study is due to clinical illness or metabolic disturbances. It certainly cannot explain the elevated levels in siblings and parents

who were without clinical signs or symptoms of illness at the time of sampling. In addition levels of cytokine production did not correlate with patients' blood glucose levels.

Cytokine production such as IL-2 and IFN-γ by T cells, after PHA stimulation was not different between family members with Type 1 diabetes, diabetic children and normal controls. These findings are in agreement with those of Toms *et al*. (1991), who found normal production of IFN-γ in patients with Type 1 diabetes after concanavalin A stimulation (a mitogen), in contrast with earlier studies by Kaye et al. (1986) and Giordano et al. (1989) who found a decreased synthesis of IL-2 by mitogen-stimulated lymphocytes from patients with Type 1 diabetes. The discrepancy between the present results and others could be explained by different experimental conditions employed, or duration of diabetes in the subjects. Other studies used either a high concentration of PHA (25 μg/ml) (Giordano et al., 1989) as compared to 1 μg/ml of PHA in present study or used patients with Type 1 diabetes of <1 year duration from diagnosis (Kaye et al., 1986) compared to the majority of patients in the present study who were >1 year from diagnosis.

In summary, hypersecretion of TNF-α and IL-1α by PBMC was present in diabetic children and is consistent with the role of macrophages in β cell damage. Hypersecretion of these cytokines was also found in family members of children with Type 1 diabetes and some of this abnormality may be linked to genes coding the HLA system some of which increase risk of Type 1 diabetes. Normal secretion of IL-2 and IFN-γ by PBMC of family members of patients with Type 1 diabetes and diabetic children indicates that T cell function is not impaired.

CHAPTER 9

CIRCULATING LEVELS OF CYTOKINES IN NON-OBESE DIABETIC (NOD) MICE

9.1 MICE

A colony of NOD mice has been established in the Comparative Biology Unit (CBU) at King's. These studies have been ratified by the CBU and are covered under the animal (Scientific Procedures) Act 1986, Project Licence number PPL 70/01555. These animals were obtained from Dr. Hattori, Joslin Diabetes Centre, Boston, U.S.A. The colony is tested weekly for urinary glucose until 30 weeks of age, by which time the incidence of diabetes is 74% in females and 47% in males.

Forty seven NOD mice with Type 1 diabetes (26 females, median age 16 weeks, range 8-28) and 37 NOD mice without diabetes (17 females), all aged 30 weeks old and in whom diabetes would now be unlikely to develop were studied. As controls, we studied 23 NODxB10 F1 generation mice (12 females, median age 15 weeks, 13-20) and 20 SJL mice (10 females) aged 15 weeks. SJL and B10 mice were chosen because they have a similar genetic background to the parent strain of NOD mice (Wicker *et al*., 1987). When B10 mice are crossed with NOD mice, the F1 generation mice has insulitis but does not develop diabetes.

9.2 RESULTS

Serum levels of TNF-α (macrophage derived cytokine), IL-2 (to denote the T_H1 cytokine profile) and IL-4 (to denote the T_H2 cytokine profile) were measured in diabetic and non-diabetic NOD mice, NODxB10 F1 mice and normal SJL mice.

9.2.1 CIRCULATING LEVELS OF CYTOKINES IN NOD MICE

TNF-α. Levels of TNF-α were significantly increased in diabetic NOD mice (median 4 pg/ml, range 0-68) when compared with non-diabetic NOD mice (0 pg/ml, 0-29, $p<0.004$), NODxB10 F1 mice (0 pg/ml, 0-8, $p<0.001$) and normal SJL mice in whom TNF-α was not detected (0 pg/ml, 0-0, $p<0.001$) (**Figure 9.1**). No significant differences exist between non-diabetic NOD mice, NODxB10 F1 mice and normal SJL mice with respect to serum levels of TNF-α. Levels of TNF-α exceeding the highest normal value (8 pg/ml) were found in 25/47 (53%) diabetic NOD mice, 7/37 (18%) non-diabetic NOD mice and 1/23 (4%) NODxB10 F1 mice.

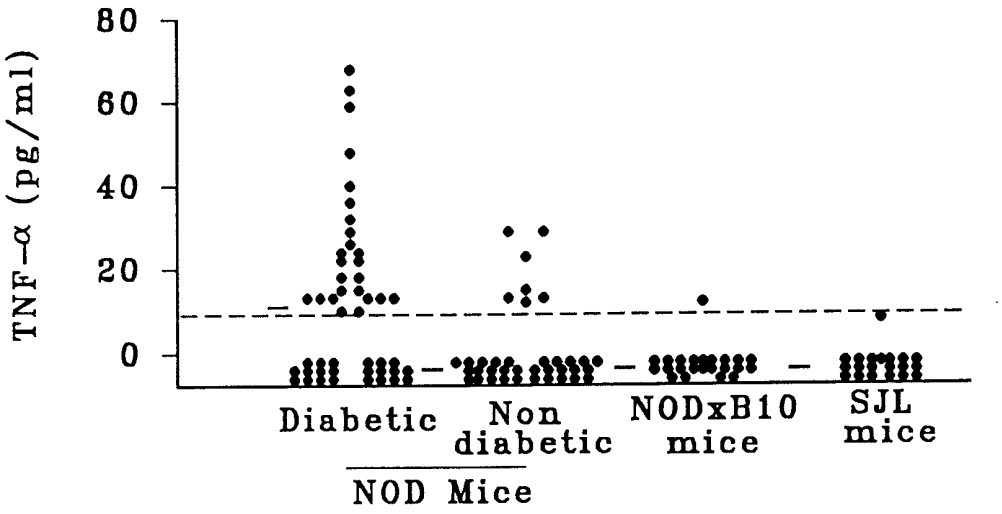


Figure 9.1. TNF-α values in the serum of diabetic and non-diabetic NOD mice, NODxB10 F1 mice and normal SJL mice. The dashed line represents the upper limit of SJL mice. Levels of TNF-α were significantly higher in diabetic NOD mice when compared with non-diabetic NOD mice ($p<0.004$), NODxB10 F1 mice ($p<0.001$) and normal SJL mice ($p<0.001$). Horizontal bars represent medians.

IL-2. Levels of IL-2 were significantly increased in diabetic NOD (median 170 pg/ml,range 0-1142) when compared with non-diabetic NOD mice (0 pg/ml, 0-525, $p<0.01$), NODxB10 F1 mice (0 pg/ml, 0-40, $p<0.001$) and normal SJL mice in whom IL-2 was not detectable (0 pg/ml, 0-0, $p<0.0001$) (**Figure 9.2**). There were no

significant differences between non-diabetic NOD mice, NODxB10 F1 mice and normal SJL with regard to serum levels of IL-2. Levels of IL-2 exceeding the highest normal value (0 pg/ml) were found in 32/47 (68%) diabetic, 7/37 (19%) non-diabetic NOD mice and 2/23 (8%) NODxB10 F1 mice.

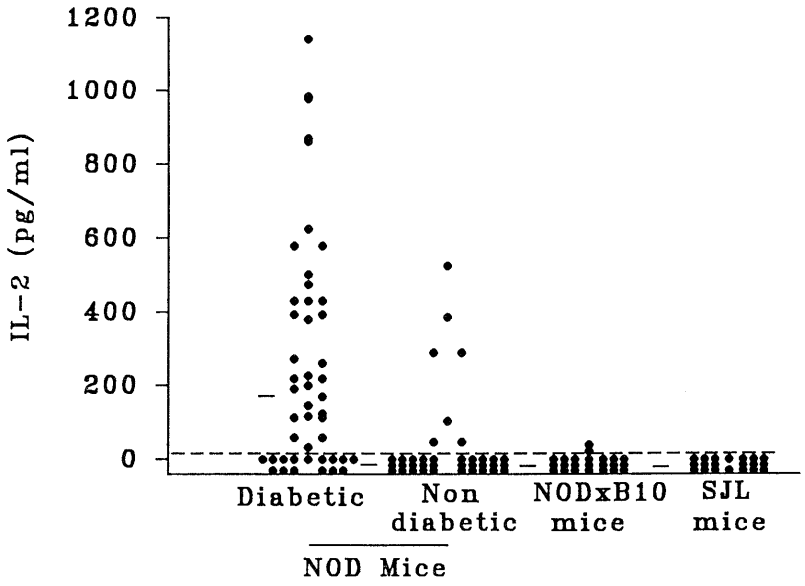


Figure 9.2. IL-2 values in the serum of diabetic and non-diabetic NOD mice, NODxB10 F1 mice and normal SJL mice. The dashed line represents the upper limit of SJL mice. Levels of IL-2 were significantly higher in diabetic NOD mice when compared with non-diabetic NOD mice ($p<0.01$), NODxB10 F1 mice ($p<0.001$) and normal SJL mice ($p<0.0001$). Horizontal bars represent medians.

IL-4. IL-4 was not detected in the serum of either test or control mice.

9.3 COMMENT

This study demonstrates that levels of TNF-α (macrophage-derived cytokine) and IL-2 (T_H1 cytokine) are elevated in diabetic NOD mice when compared with non-diabetic NOD mice, NODxB10 F1 mice and normal SJL mice. The presence of this profile of cytokines implies activation of the T_H1 subset of helper cells at diagnosis of Type 1 diabetes. In contrast, elevated levels of IL-4 (T_H2 cytokine) were not detectable in the serum of either test or controls mice, indicating a lack of recruitment of T_H2 cells at diagnosis of diabetes.

The finding of high levels of IL-2 but not IL-4 in our study indicates that a cellular immune response involving T_H1 cells characterises the onset of diabetes in this animal model as discussed in section 5.3.

Increased levels of TNF-α in diabetic NOD mice give support to a critical role for macrophages in the pathogenesis of diabetes in this model as discussed (vide 5.7). It has been suggested that the release of TNF-α by cells infiltrating the pancreatic islets is a major effector mechanism of tissue destruction in Type 1 diabetes (Bendtzen, 1989).

Increased levels of cytokines in this animal model of Type 1 diabetes could be due to either environmental factors or be genetically determined. It is unlikely that high levels of cytokines in the present study are solely determined by genetic factors since levels of IL-2 and TNF-α in non-diabetic NOD mice or NODxB10 F1 mice were within the normal range. It is likely therefore that high levels of cytokines are related to immune processes triggered by environmental factors such as a viral infection, as discussed before (vide 5.3).

In conclusion, the results in this section demonstrate that cytokines produced by macrophages (TNF-α) and T_H1 cells (IL-2) are elevated in association with the development of diabetes in this animal model of Type 1 diabetes and argue for a T_H1-directed process of β cell damage, with the involvement of activated macrophages.

CHAPTER 10

STUDIES OF CELLULAR EXPRESSION OF TNF-α AND IFN-γ IN PANCREATIC SECTIONS FROM NON-OBESE DIABETIC (NOD) MICE

10.1 MICE

A total of 39 mice were studied. Nineteen NOD mice of various ages were examined comprising 10 diabetics (6 females, median age 16 weeks, range 8-23) and 9 NOD mice at a pre-diabetic stage (7 females, median age 10 weeks, 4-15) (colony incidence >70%). As controls, 10 NODxB10 F1 mice (5 females, median age of 15 weeks, 13-20) and 10 SJL control mice (5 females) at age of 15 weeks were also studied.

Mice were sacrificed and pancreata were removed from all animals. Serial sections of the blocks were cut and consecutive sections were examined for cytokine producing cells. At least three islets were counted for the positive staining of cytokine producing cells. The mononuclear cell (MNC) infiltrate was characterised in pancreas sections using three grades of severity: no infiltration; a small number of infiltrating mononuclear cells at the islet periphery but covering <20% of the perimeter (moderate); islet perimeter completely surrounded by a mantle of mononuclear cells (severe). All diabetic pancreas sections had severe islet lymphocytic infiltrate.

10.2 RESULTS

Cellular expression of cytokines such as TNF-α and IFN-γ was examined in islets of Langerhans of NOD mice with diabetes and those at a pre-diabetic stage, NODxB10 F1 mice and normal SJL mice using indirect immunohistochemical or immunofluorescence techniques.

10.2.1 EXPRESSION OF IFN-γ AND TNF-α IN ISLETS OF LANGERHANS

Using an indirect immunoperoxidase technique, IFN-γ producing cells were detected within the inflammatory cell infiltrate in pancreas sections of diabetic mice (**Figure 10.1**). No staining was detected for TNF-α producing cells within the inflammatory cell infiltrate. However, positive staining for TNF-α was detected within islet cells (**Figure 10.2**) and the latter pattern was confirmed using an immunofluorescence technique (**Figure 10.3**).

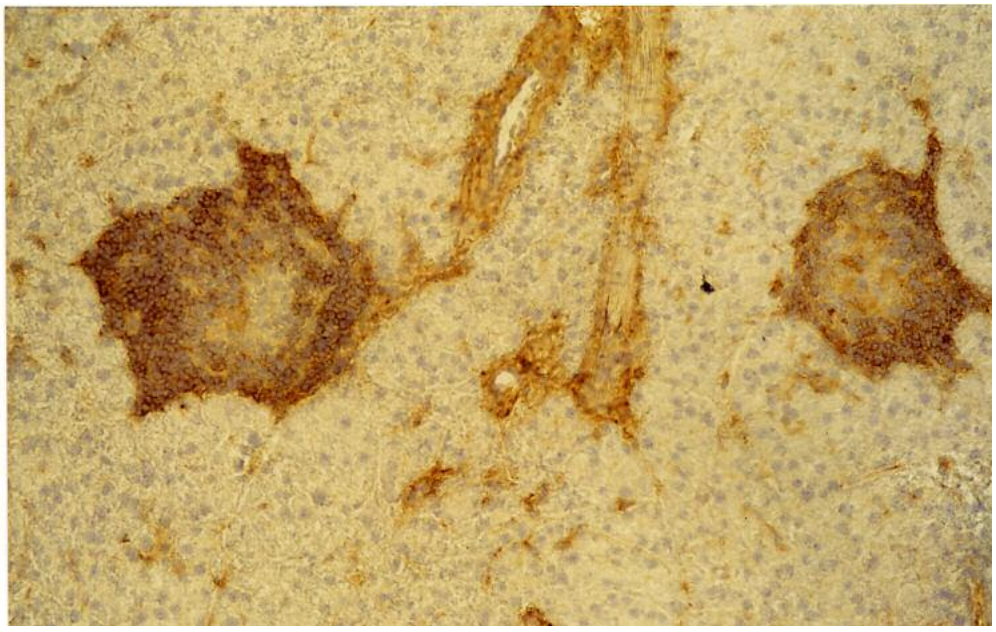


Figure 10.1. Pancreatic section from diabetic NOD mice showing mononuclear cells with a diffuse cytoplasmic staining of IFN-γ (stained dark brown). Positively stained cells are mainly surrounding islet β cells. (immunoperoxidase staining, original magnification 25x)

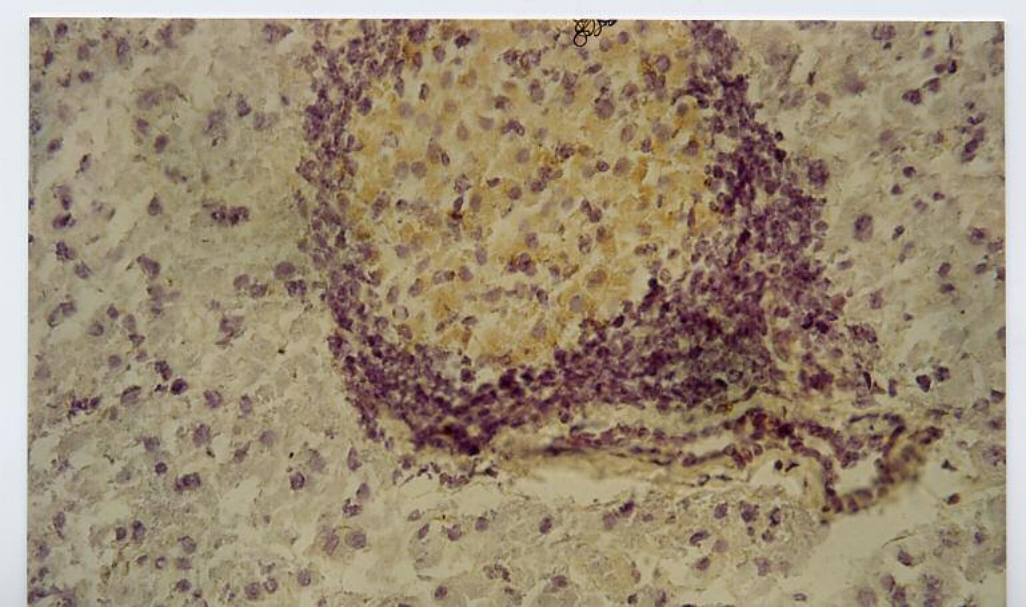


Figure 10.2. Pancreatic section from diabetic NOD mice showing islet cells staining with TNF-α (stained weak brown or yellow) (immunoperoxidase staining, original magnification 40x)

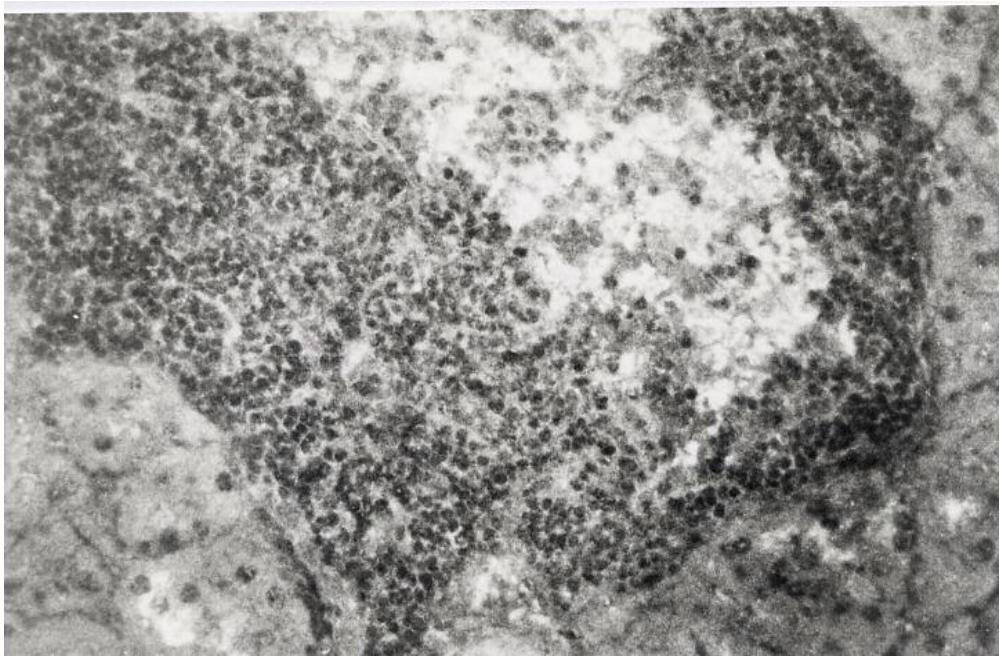


Figure 10.3. Pancreatic section from diabetic NOD mice showing islet cells staining of TNF-α (bright fluorescing) and surrounding negative staining of mononuclear cell infiltration (black cells). (Immunofluorescence staining, original magnification 40x)

9 of 10 diabetic NOD mice were positive for IFN-γ and 8 for TNF-α. Both cytokines were detected simultaneously in eight and the score of the expression of TNF-α correlated with the score of the expression of IFN-γ ($r=0.76$, $p<0.01$) (**Table 10.1**). Expression of TNF-α and IFN-γ was also detected in NOD mice at the pre-diabetic stage (**Figure 10.4**). 7 of 9 pre-diabetic NOD mice were positive for IFN-γ and five for TNF-α. None of the sections from control mice, whether normal or with insulitis, was positive for either cytokine. The frequency of expression of TNF-α and IFN-γ was significantly higher in diabetic NOD mice than control mice with insulitis ($\chi^2= 5.7$, $p<0.04$ for both cytokines).

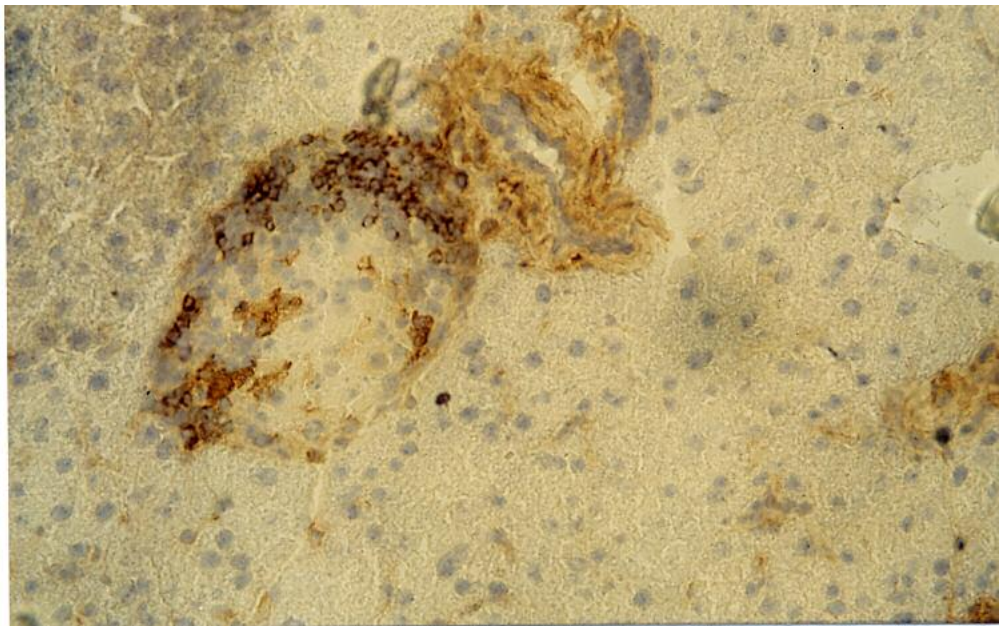


Figure 10.4. Pancreatic section from a NOD mouse at the pre-diabetic stage (10 weeks old) showing mononuclear cells with a diffuse cytoplasmic staining of IFN-γ (stained dark brown). Positively stained cells are mainly surrounding islet cells. (immunoperoxidase staining, original magnification 25x)

Table 10.1 Age, sex, immunological and histological details of NOD mice.

NOD mice	**Sex**	**Age (wks)**	**Islet cell infiltrate**	**TNF-α**	**IFN-γ**
Diabetic					
1	F	20	Severe	1	3
2	F	8	Severe	2	3
3	F	16	Severe	2	2
4	F	20	Severe	3	3
5	F	12	Severe	0	1
6	M	20	Severe	0	0
7	M	16	Severe	1	2
8	M	12	Severe	2	3
9	M	23	Severe	1	2
10	M	16	Severe	2	2
"Pre-diabetic"					
1	F	4	Negative	0	0
2	F	8	Moderate	0	1
3	F	10	Severe	2	2
4	F	15	Severe	0	2
5	F	15	Severe	2	2
6	M	4	Moderate	0	0
7	M	8	Moderate	2	2
8	M	10	Severe	1	2
9	M	15	Severe	1	2

10.3 COMMENT

The results in this section demonstrate that both IFN-γ and TNF-α producing cells are present in the islet of Langerhans of diabetic NOD mice. Cells with positive staining for IFN-γ are detected within the inflammatory cell infiltrate, while TNF-α staining is found within islet cells. None of the sections from SJL mice or NODxB10 F1 mice were positive for either cytokine suggesting that these mediators may play a role in β cell damage in diabetics as already discussed (vide 5.3).

There has been much interest in the possible roles of IL-1 and TNF-α, both macrophage products, in the pathogenesis of Type 1 diabetes (Mandrup-Poulsen et al. 1987b; Pujol-Borrell et al., 1987). TNF-α producing cells associate the inflammatory cell infiltrate were not detectable but the possibility that macrophages have a critical role in the disease process in this animal model of diabetes can not be excluded. The finding of positive staining for TNF-α apparently within the islet cells in present study gives support to the hypothesis that β cells are able to produce cytokines. Evidence to support this concept comes from in vitro experiments shows that pancreatic β cells produce TNF-α after exposure to IL-1 and IFN-γ (Yamada et al., 1993) and IL-6 (Campbell et al., 1989) and that this production is up-regulated when cytokines are given in combination.

The fact that the presence of TNF-α producing-cells strongly correlates with that of IFN-γ producing-cells suggests that these cytokines may act pathogenically in concert by enhancing/inducing MHC class II molecules, a critical step in the process of antigen recognition, and by recruiting inflammatory cells to the site of the lesion (Foulis et al., 1991). Although mice with insulitis have an inflammatory cell infiltrate of a severity similar to that in diabetic mice, they did not have positive staining for TNF-α or IFN-γ, suggesting that these cytokines may be closely related to the autoimmune attack.

The view that cytokine producing cells are involved in the pathogenic process of autoimmune Type 1 diabetes is supported by observations that in other autoimmune diseases, such as the autoimmune form of hepatitis (vide section 12) and rheumatoid arthritis, TNF-α and IFN-γ producing-cells are prominent in the characteristic inflammatory lesions in the liver and synovium respectively (Hussain et al., in press; Chu et al., 1991).

In conclusion, the presence of IFN-γ and TNF-α producing cells in the inflamed islet in diabetic NOD mice and not in animals without the disease implies that cytokines may be involved in the autoimmune process of Type 1 diabetes. The expression of TNF-α within islet cells supports the hypothesis that β cell destruction

and MHC class II antigen expression in autoimmune diabetes may be attributable to endogenous cytokine production by β-cells.

CHAPTER 11

STUDIES OF CYTOKINE LEVELS IN AUTOIMMUNE HEPATITIS, AN ORGAN-SPECIFIC AUTOIMMUNE DISEASE

11.1 PATIENTS

A total of 67 patients (39 females, median age 11 years, range 1-17) were studied. Twenty-three patients had autoimmune hepatitis (AIH) (19 females, median age 12 years, range 2-17) and were diagnosed according to internationally agreed criteria (Scheuer, 1991) (**Table 11.1**). Five of these were positive for anti-nuclear antibody (ANA) and smooth muscle antibody (SMA), one for ANA and liver kidney microsomal antibody (LKM), 4 for ANA alone, 8 for SMA alone and 1 for LKM. Eight patients were tested before immunosuppressive therapy and had high values of aspartate aminotransferase (AST), immunoglobulin G (IgG) and non-organ specific autoantibodies. Fifteen were tested while on immunosuppressive treatment (Prednisolone 0.5-2 mg/kg/day; azathioprine 1-1.5 mg/kg/day). Four of them had signs of active disease (defined by having severe portal and peri-portal tract infiltrate) with elevated values of AST, IgG and autoantibodies. The remainder had no biochemical, immunological or histological evidence of active disease. Seven of them had associated cirrhosis.

Eleven children had autoimmune sclerosing cholangitis (ASC) (6 females, median age 11 years, range 5-15) (**Table 11.2**). All had characteristic endoscopic retrograde cholangio-pancreatographic changes (El-Shabrawi et al., 1987). Six were positive for SMA and ANA, 2 for SMA only and one for ANA only. Seven were tested at diagnosis before therapy and all had active disease. Four were tested while on treatment with salazopyrine 30 mg/kg/day and prednisolone 0.5 mg/kg/day. Three patients had signs of active disease with high values of AST and IgG and associated cirrhosis.

As pathological controls, 35 children with other liver disorders due to metabolic causes, including Wilson's disease (WD, progressive liver disorder associated with abnormality of copper metabolism) and alpha-1-antitrypsin deficiency (A1ATD, progressive liver disorder associated with abnormal alpha 1 phenotype causing deficiency of A1AT) were studied. 11 had Wilson's disease (2 females, median age 16 years, range 8-19) (**Table 11.3**). All patients were tested while on treatment with penicillamine 20 mg/kg/day and only one had histological evidence of active disease. Twenty-four patients had A1ATD (12 females, median 7 years, 1-14) (**Table 11.4**). Twenty-one patients were receiving no treatment; 10 had signs of active disease and eight had cirrhosis. The remainder were tested while receiving treatment with colchicine 25 μg/kg/day and/or penicillamine 20 mg/kg/day. These children were selected because their liver biopsy samples showed degrees of portal tract infiltration and piecemeal necrosis similar to those observed

in chronic autoimmune hepatitis.

We also studied 25 healthy children as controls (18 females, median age 8 years, range 2-17) who were recruited by the Medway and Gillingham Branch (Kent) of the Children's Liver Disease Foundation (CLDF), and gave blood after informed consent from themselves or their parents, as appropriate.

Table 11.1 Clinical, biochemical, immunological and histological details of the patients with autoimmune hepatitis at the time of testing.

Patien-ts no.	Sex	Age (yrs)	Disease status[A]	Immunosup-pressive treatment[*]	Cirrho-sis	AST[**] (IU/l)	IgG[***] g/l	Autoantibodies[#] SMA ANA LKM
1	M	12	Active	none	Present	490	32.7	320 20 Neg
2	F	13	Active	none	Absent	840	50.3	Neg 640 Neg
3	F	5	Active	none	Absent	1480	18.2	20 160 Neg
4	F	5	Active	none	Absent	185	20.6	Neg 160 640
5	F	8	Active	none	Present	222	18	40 Neg Neg
6	F	11	Inactive	Pred	Present	34	ND	ND ND ND
7	F	14	Active	Pred	Present	465	35.5	160 Neg Neg
8	F	11	Inactive	Pred/Aza	Present	66	24.5	Neg Neg 160
9	F	9	Active	none	Present	930	39	640 Neg Neg
10	M	15	Inactive	Pred	Present	55	12.5	160 Neg Neg
11	F	5	Active	none	Present	293	37.4	Neg 40 Neg
12	F	13	Active	none	Present	100	ND	640 Neg Neg
13	M	12	Inactive	Pred	Present	46	16.4	10 20 Neg
14	F	2	Active	Pred	Present	114	20.8	Neg 10 Neg
15	F	12	Active	Pred/Aza	Present	139	9.7	10 Neg Neg
16	F	12	Inactive	Pred	Absent	21	ND	ND ND ND
17	F	12	Inactive	Pred	Present	35	36.4	ND ND ND
18	F	17	Inactive	Pred	Present	55	22.7	160 Neg Neg
19	F	9	Inactive	Pred	Present	15	12.7	20 10 Neg
20	M	7	Active	Pred	Absent	89	10.5	40 Neg 640
21	F	13	Inactive	Pred/Aza	Absent	63	13.5	160 Neg Neg
22	F	17	Inactive	Pred/Aza	Absent	59	10.9	10 20 Neg
23	F	6	Inactive	Pred	Absent	32	10.5	Neg 40 Neg
Overa-ll group	19F:4M	12 (2-17)	12A:11In	15Tr:8Untr	15P:8A	89 (15-1480)	19 (9.7-50)	14/19 9/19 2/19

[A]Disease status; patients with active disease were chosen on the basis that they had histological and/or biochemical evidence of disease activity at time of study. * Treatment; Pred=prednisolone, Aza=azathioprine; ** Normal value < 45 IU/l; *** Normal values for children aged between 2 and 5 years are >5 and < 17 g/l; 5 and 16 years are >7 and <18 g/l. # Autoantibodies were detected on rat tissue by indirect immunofluorescence. SMA =anti-smooth muscle antibody; ANA =anti-nuclear factor antibody; LKM =anti-liver kidney microsomal antibody.

Table 11.2 Clinical, biochemical, immunological and histological details of patients with autoimmune sclerosing cholangitis at the time of testing.

Patients no.	Sex	Age (yrs)	Disease status[A]	Immunosuppressive treatment[*]	Cirrhosis	AST[**] (IU/l)	IgG[***] g/l	Autoantibodies[#] SMA ANA LKM
1	M	11	Active	None	Present	125	17.2	Neg Neg Neg
2	F	13	Active	Pred/SLZ	Present	178	11.6	640 640 Neg
3	F	7	Active	None	Present	93	8.8	40 640 Neg
4	M	5	Active	None	Absent	120	16.4	160 Neg Neg
5	M	14	Active	None	Present	389	29.4	640 640 Neg
6	F	8	Active	None	Absent	177	16.2	160 40 Neg
7	F	12	Active	None	Absent	504	42.3	640 40 Neg
8	F	7	Inactive	Pred/SLZ	Absent	49	16.2	10 Neg Neg
9	M	8	Active	None	Present	174	70.8	40 640 Neg
10	M	14	Inactive	Pred/SLZ	Absent	18	20.9	Neg 40 Neg
11	F	15	Active	Pen	Present	103	13.9	40 Neg Neg
Overall group	6F:5M	11 (5-15)	9A:2In	4Tr:7Untr	6P:5A	120 (18-504)	16.4 (8.8-70.8)	9/11 7/11 0/0

[A]Disease status; patients with active disease were chosen on the basis that they had histological and/or biochemical evidence of disease activity at time of study. * Treatment: Pred=prednisolone; SLZ=salazopyrine; Pen=penicillamine. ** Normal values < 45 IU/l. *** Normal value for children aged between 2 and 5 years are >5 and <17 g/l, and 5 and 16 years are >7 and <18 g/l. # Autoantibodies as detected on rat tissue by indirect immunofluorescence. SMA =anti-smooth muscle antibody; ANA =anti-nuclear factor antibody; LKM =anti-liver kidney microsomal antibody.

Table 11.3 Clinical, biochemical, immunological and histological details of the patients with Wilson's disease at the time of testing.

Patients no.	**Sex**	**Age (yrs)**	**Disease status[A]**	**Treatment[*]**	**Cirrhosis**	**AST[**] (IU/l)**
1	M	10	Active	Penc	Present	290
2	F	8	Inactive	Penc	Present	51
3	M	11	Inactive	Penc	Absent	32
4	M	19	Inactive	Penc	Absent	38
5	M	14	Inactive	Penc	Present	36
6	M	16	Inactive	Penc	Present	33
7	M	12	Inactive	Penc	Absent	22
8	M	16	Inactive	Penc	Present	34
9	M	23	Inactive	Penc	Absent	32
10	F	21	Inactive	Penc	Absent	62
11	M	19	Inactive	Penc	Absent	33
Overall group	2F:9M	16 (8-23)	1A:10In	11Tr:0Untr	5P:6A	34 (22-290)

[A]Disease status; patients with active disease were chosen on the basis that they had histological and/or biochemical evidence of disease activity at time of study. * Treatment; Pen= penicillamine. ** Normal value <45 IU/l

Table 11.4 Clinical, biochemical, immunological and histological details of the patients with α-1-anti-trypsin deficiency (A1ATD) at the time of testing.

Patients no.	Sex	Age (yrs)	Disease status[A]	Treatment*	Cirrhosis	AST** IU/l
1	F	2	Inactive	None	Absent	42
2	M	3	Inactive	None	Absent	47
3	M	2	Active	None	Present	770
4	F	1	Active	None	Present	232
5	M	7	Inactive	None	Absent	53
6	F	10	Inactive	Col	Present	50
7	M	14	Inactive	None	Absent	52
8	F	14	Active	Penc	Present	102
9	M	8	Inactive	Col	Present	38
10	M	2	Active	None	Present	98
11	F	3	Active	None	Absent	60
12	M	3	Inactive	None	Present	58
13	F	3	Active	None	Absent	87
14	M	11	Active	None	Present	133
15	F	12	Inactive	None	Absent	33
16	F	4	Active	None	Present	302
17	F	4	Active	None	Present	121
18	M	6	Active	None	Present	261
19	F	6	Inactive	None	Absent	45
20	F	7	Active	None	Present	162
21	M	9	Inactive	None	Absent	48
22	F	7	Inactive	None	Absent	37
23	M	8	Inactive	None	Absent	45
24	M	9	Inactive	None	Absent	48
Overall group	12F: 12M	7 (1-14)	11A:13In	3Tr:21Un-tr	12P:12A	59 (37-770

[A]Disease status; patients with active disease were chosen on the basis that they had histological and/or biochemical evidence of disease activity at time of study. * Treatment: Col= colchicine; Pen= penicillamine ** Normal value < 45 IU/l

11.2 RESULTS

Serum levels of TNF-α, IL-1α, IL-6 (macrophage-derived cytokine), IFN-γ (T_H1-derived cytokine), IL-4 (T_H2-derived cytokines) and sIL-2R were measured in children with autoimmune liver disease (AIH and ASC) and those with liver disorders due to metabolic causes, namely Wilson's disease and alpha 1 antitrypsin deficiency at various disease stages, as well as control subjects.

11.2.1 MACROPHAGE-DERIVED CYTOKINES

TNF-α. Levels of TNF-α were significantly higher in children with AIH (median 7 pg/ml, range 0-132, $p<0.003$) and A1ATD (median 8 pg/ml, range 0-51, $p<0.007$) when compared with normal controls (median 0 pg/ml, range 0-10) (**Figure 11.1**). In children with AIH, ASC and A1ATD, levels of TNF-α were significantly higher in patients with active than inactive disease ($p<0.01$). Levels of TNF-α exceeding the highest normal value (>10 pg/ml) were found in 9/21 (43%) children with AIH, 5/11 (46%) with ASC, 1/8 (13%) with WD and 7/21 (33%) with A1ATD.

IL-1α. Levels of IL-1α were significantly elevated in children with AIH (median 170 pg/ml, range 38-1980, $p<0.002$), ASC (median 393 pg/ml, range 30-1980, $p<0.002$), WD (median 60 pg/ml, range 20-1900, $p<0.03$) and A1ATD (median 275 pg/ml, range 60-994, $p< 0.003$) when compared to normal controls (median 50 pg/ml, range 30-192) (**Figure 11.2**). In children with AIH and ASC, levels of IL-1α were significantly higher in patients with active than inactive disease ($p<0.01$). Levels of IL-1α exceeding the highest normal value (>192 pg/ml) were found in 7/23 (30%) children with AIH, 7/11 (64%) with ASC, 4/11 (35%) with WD and 12/21 (57%) with A1ATD.

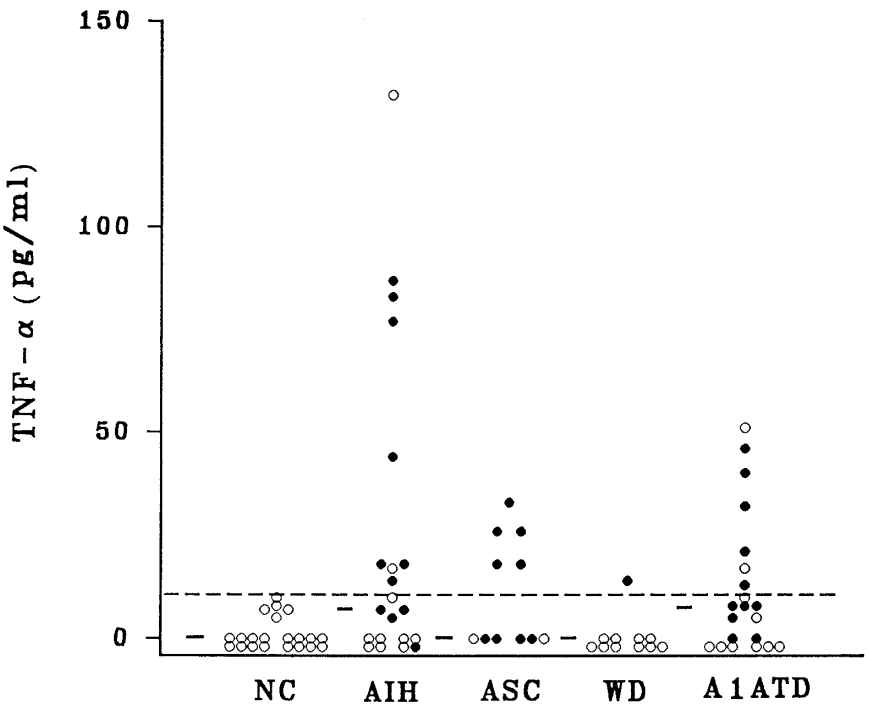


Figure 11.1. TNF-α levels in the serum of children with different chronic liver diseases. The dashed line represents the upper limit of normal children. Levels of TNF-α were significantly higher in children with autoimmune hepatitis (AIH) ($p<0.003$) and alpha-1-antitrypsin deficiency (A1ATD) ($p<0.007$) when compared with normal children (NC). In children with AIH, autoimmune sclerosing cholangitis (ASC) and A1ATD, levels of TNF-α were significantly higher in patients with active (●) than inactive disease (○) ($p<0.01$). Horizontal bars represent medians.

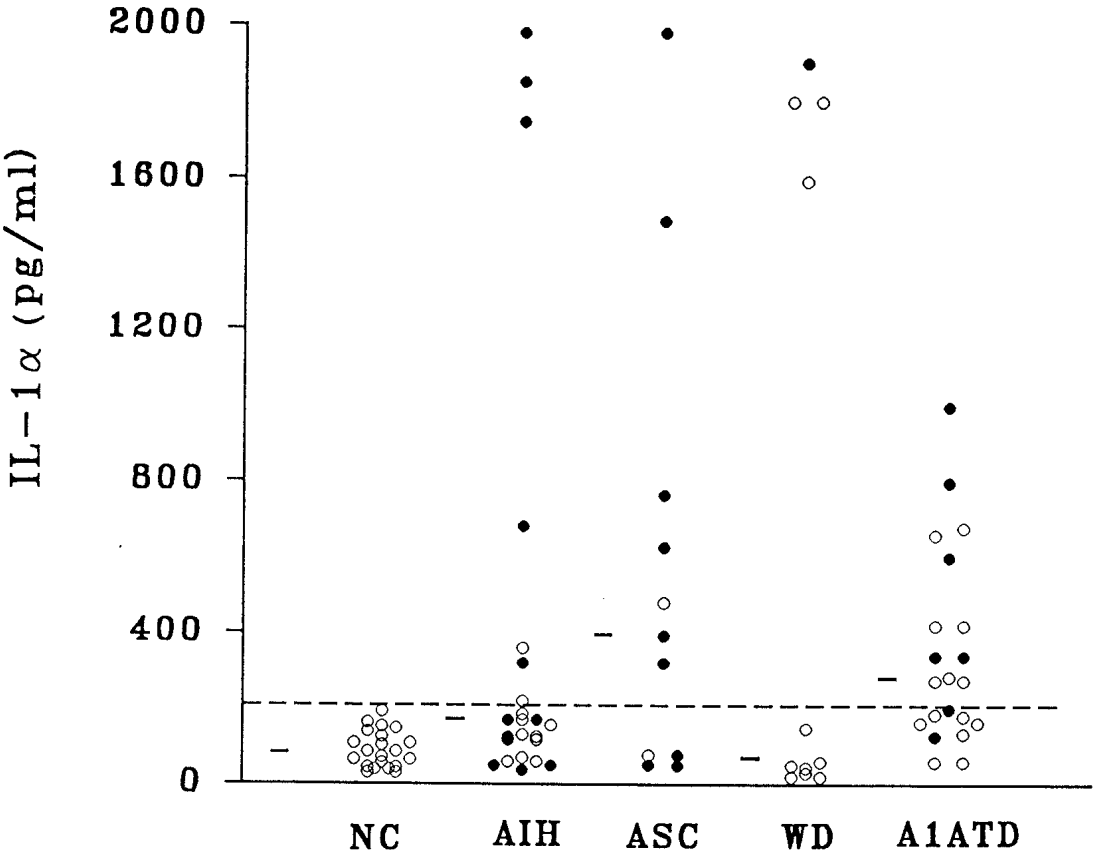


Figure 11.2. IL-1α levels in the serum of children with different chronic liver disease. The dashed line represents the upper limit of normal children. Levels of IL-1α were significantly higher in children with autoimmune hepatitis (AIH) (p<0.002), autoimmune sclerosing cholangitis (ASC) (p<0.002), Wilson's disease (WD) (p<0.03), and alpha-1-antitrypsin deficiency (A1ATD) (p<0.003) when compared with normal children (NC). In children with AIH and ASC, levels of IL-1α were significantly higher in patients with active (●) than inactive disease (○) (p<0.01). Horizontal bars represent medians.

IL-6. Levels of IL-6 were significantly increased in children with AIH (median 28 pg/ml, range 0-55, p<0.001), ASC (median 23 pg/ml, range 0-65, p< 0.02) and WD (median 0 pg/ml, range 0-34, p<0.03) when compared with normal controls (median 0 pg/ml, range 0-10) (**Figure 11.3**). In children with A1ATD, levels of IL-6 were significantly higher in patients with active than inactive disease (p<0.01). Levels of IL-6 exceeding the highest normal value (>10 pg/ml) were found in 14/21 (67%) children with AIH, 6/11 (55%) with ASC, 5/11 (46%) with WD and 8/21 (38%) with A1ATD.

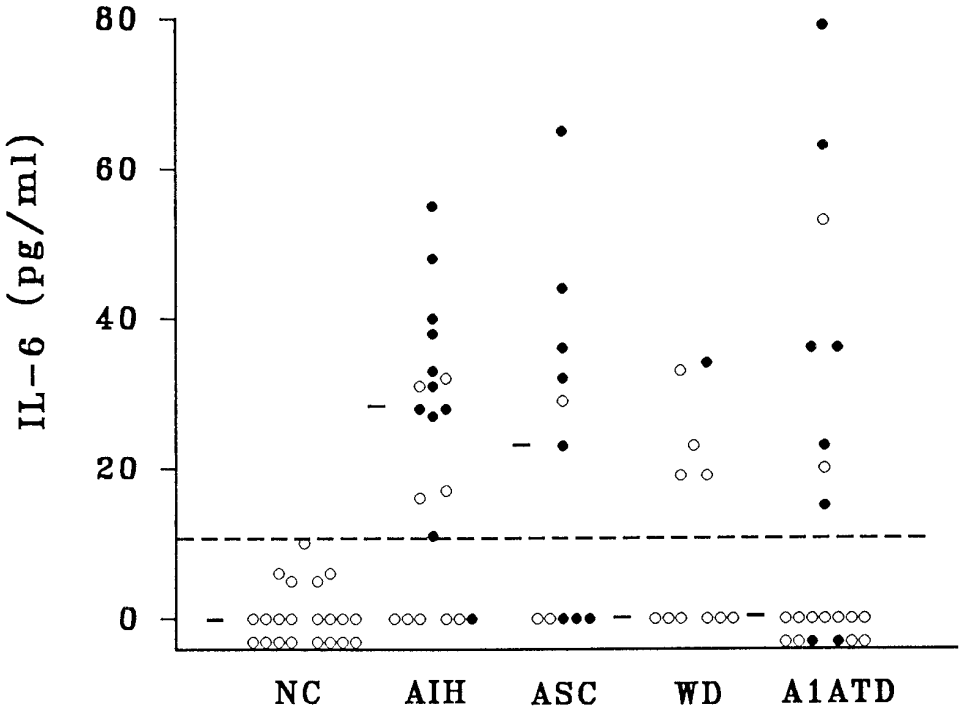


Figure 11.3. IL-6 levels in the serum of children with different chronic liver diseases. The dashed line represents the upper limit of normal children. Levels of IL-6 were significantly higher in children with autoimmune hepatitis (AIH) ($p<0.001$), autoimmune sclerosing cholangitis (ASC) ($p<0.02$), Wilson's disease (WD) ($p<0.03$) when compared with normal children (NC). In children with alpha-1-antitrypsin deficiency (A1ATD), levels of IL-6 were significantly higher in patients with active (●) than inactive disease (○) ($p<0.01$). Horizontal bars represent medians.

11.2.2 T_H1 PROFILE

IFN-γ. Cytokines produced by T cells such as IFN-γ were also measured. Levels of IFN-γ were significantly increased only in children with AIH (median 310 pg/ml, range 0-3500, $p<0.04$) when compared with normal controls (median 0, range 0-250) (**Figure 11.4**). Levels of IFN-γ were not different between ASC, WD, A1ATD and NC. In children with A1ATD, levels of IFN-γ were significantly increased in patients with active disease compared to those with inactive ($p<0.01$). Levels of IFN-γ exceeding the highest normal value (>250 pg/ml) were found in 11/21 (52%) AIH, 4/11 (36%) ASC, 3/11 (27%) WD and 9/21 (43%) A1ATD.

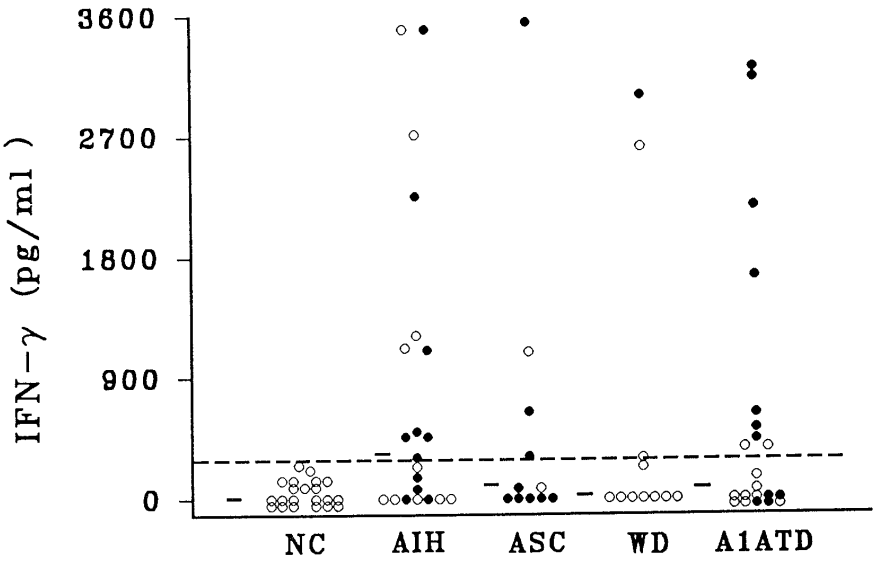


Figure 11.4. IFN-γ levels in the serum of children with different groups of chronic liver disease. The dashed line represents the upper limit of normal children. Levels of IFN-γ were significantly higher in children with autoimmune hepatitis (AIH) when compared with normal children (NC) ($p<0.04$). In children with alpha-1-antitrypsine deficiency (A1ATD), levels of IFN-γ were significantly increased in patients with active (●) than inactive disease (○) ($p<0.01$). Autoimmune sclerosing cholangitis=ASC and Wilson's disease=WD. Horizontal bars represent medians.

11.2.3 T_H2 PROFILE

IL-4. The median levels of IL-4 were not different in children with AIH, ASC, WD and A1ATD when compared with normal controls. Levels of IL-4 exceeding the highest normal value (30 pg/ml) were found in 3/21 (14%) children with AIH, 1/11 (8%) with ASC, 0/11 (0%) with WD, and 0/21 (0%) with A1ATD.

11.2.4 LEVELS OF sIL-2R IN CHILDREN WITH AUTOIMMUNE CHRONIC LIVER DISEASE

sIL-2R. Significantly increased levels of sIL-2R were observed only in patients with autoimmune liver diseases; AIH (median 615 U/ml, range 50-2970, $p<0.003$) and ASC (median 585 U/ml, range 210-3500, $p<0.002$) when compared with normal controls (median 220 U/ml, range 115-455) (**Figure 11.5**). Levels exceeding the highest normal value (>455 U/ml) were found in 12/21 (57%) children with AIH, 9/11 (81%) with ASC, 3/11 (27%) with WD and 4/21 (19%) with A1ATD.

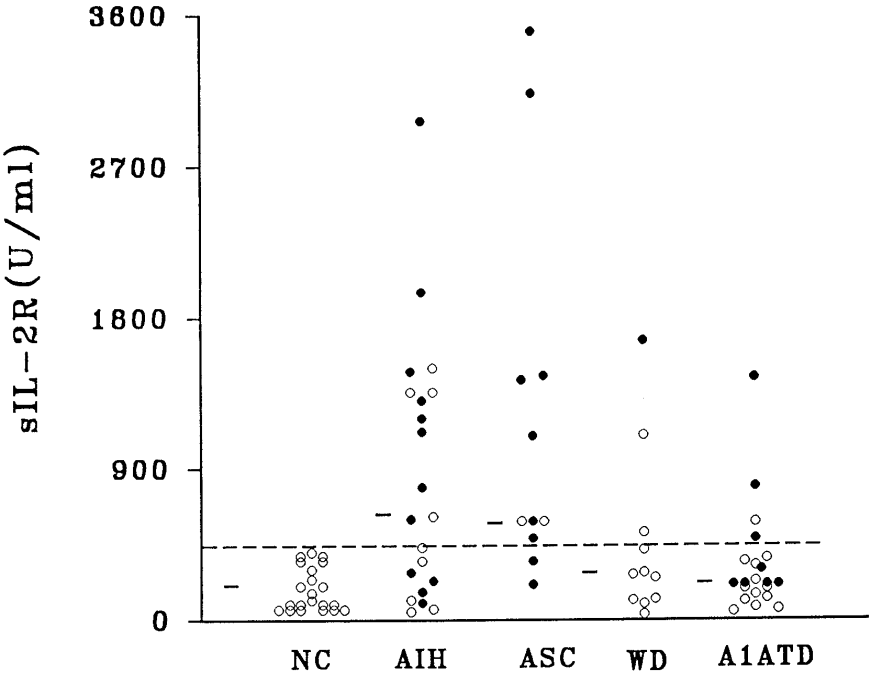


Figure 11.5. sIL-2R levels in the serum of children with different chronic liver diseases. The dashed line represents the upper limit of normal children. Levels of sIL-2R were significantly higher in children with autoimmune hepatitis (AIH) ($p<0.003$) and autoimmune sclerosing cholangitis (ASC) ($p<0.002$) when compared with normal children (NC). Levels of sIL-2R were not different between patients with active (●) and inactive disease (○) in each group of patients studied. Horizontal bars represent medians.

11.2.5 ASSOCIATION BETWEEN LEVELS OF CYTOKINES, sIL-2R AND DISEASE STAGE

Levels of TNF-α, IL-1α, IL-6 and sIL-2R were significantly higher in patients with cirrhosis (n=37) than non-cirrhotic patients (n=34) ($p<0.02$ for TNF-α, $p<0.007$ for IL-1α, $p<0.005$ for IL-6, and $p<0.01$ for sIL-2R) (**Table 11.5)**. There was no significant difference in the levels of IL-4 and IFN-γ between patients with and without cirrhosis.

Table 11.5 Levels of cytokines and sIL-2R in patients with chronic liver disease (all groups) according to presence or absence of cirrhosis.

Cytokines	with cirrhosis median range	Without median range	P values
TNF-α (pg/ml)	10 (0-132)	0 (0-77)	0.02
IL-1α (pg/ml)	330 (0-1980)	147 (0-1487)	0.007
IL-6 (pg/ml)	27 (0-78)	0 (0-53)	0.005
IFN-γ (pg/ml)	470 (0-3500)	0 (0-3500)	NS*
sIL-2R (U/ml)	485 (65-3135)	251 (33-3500)	0.01

*NS=not significant

11.2.6 ASSOCIATION BETWEEN LEVELS OF CYTOKINES AND sIL-2R AND DISEASE ACTIVITY

Levels of TNF-α, IL-1α, IL-6, IFN-γ and sIL-2R were significantly higher in patients with active disease (n=32) than patients with inactive disease (n=37) ($p<0.003$ for TNF-α, $p<0.02$ for IL-1, $p<0.005$ for IL-6, $p<0.03$ for IFN-γ and $p<0.01$ for sIL-2R) (**Table 11.6**).

Table 11.6. Levels of cytokines and sIL-2R in patients with chronic liver disease (all groups) according to the disease activity (active or inactive).

Cytokines	Active disease median range	Inactive disease median range	P values
TNF-α (pg/ml)	14 (0-87)	0 (0-132)	0.003
IL-1α (pg/ml)	340 (38-1980)	163 (20-1800)	0.02
IL-6 (pg/ml)	32 (0-79)	0 (0-53)	0.005
IFN-γ (pg/ml)	440 (0-3500)	0 (0-3470)	0.03
sIL-2R (U/ml)	695 (100-3500)	227 (33-1500)	0.01

11.2.7 ASSOCIATION BETWEEN CYTOKINES AND IgG LEVELS IN SUBJECTS STUDIED

Levels of cytokines or sIL-2R were not correlated with each other in any group studied. No correlation was found between levels of cytokines or sIL-2R and IgG values in any group studied.

11.3 COMMENT

The results in this section show that serum levels of the cytokines IL-1α, IL-6 and TNF-α are significantly elevated in patients with various forms of chronic liver disease, including those with autoimmune hepatitis, when compared with normal controls. Levels of IFN-γ and sIL-2R were significantly increased in patients with autoimmune hepatitis when compared with other forms of liver disease and normal controls. In contrast, levels of IL-4 were not different between patients with various forms of chronic liver disease and normal controls. Levels of IL-1α, IL-6, TNF-α and sIL-2R were significantly higher in patients with cirrhosis than in patients without. A similar picture was found for disease activity and showed that levels of IL-1α, IL-6, TNF-α and sIL-2R are significantly higher in patients with active disease than those with inactive disease.

The results observed confirm the finding of Tilg et al. (1992) that levels of IL-1, IL-6, TNF-α and IFN-γ are elevated in patients with various forms of chronic liver disease, including autoimmune liver disease and metabolic liver disease. That study also demonstrated higher levels of cytokines in patients with cirrhosis than in those without. Increased levels of cytokines were also reported in various liver diseases in earlier studies by Devière et al. (1990) who found increased levels of TNF-α, IL-1α and IL-6 in 50% of patients with stable alcoholic liver cirrhosis and by Khoruts et al. (1991) who found increased plasma concentrations and in vitro production of TNF-α in patients with chronic hepatitis B virus infection.

Increased levels of cytokines in chronic liver diseases of various aetiologies could be the consequence of different mechanisms. Firstly, following injury the liver shows an immense ability for regeneration. Regeneration of the liver is controlled by cytokines (Andus *et al*., 1991). Stimulatory and inhibitory effects on hepatocyte growth have been described for TNF-α, IL-1 and IL-6. However, since the data are contradictory (Feingold *et al*., 1988; Nakamura *et al*., Arakaki and Ichihara, 1988; Kuma et al., 1990; Cornell *et al*., 1990), the role of cytokines in vivo remains to be determined. Secondly, the liver appears to represent a major site of cytokine clearance (Andus *et al*., 1991). Cytokines are cleared from the plasma by the liver within a few minutes, as has been shown for IL-1β (Klapproth et al., 1989), IL-3 (Metcalf and Nicola, 1988), IL-6 (Castell et al., 1988; Sonne et al., 1990), TNF-α (Ferraiolo et al., 1988) and IFN-γ (Diez *et al*., 1987). Thus, elevated levels of endogenous cytokines in the present study could be a consequence of chronic liver failure resulting in an impairment of cytokine clearance.

Cytokine release is a sign of activation of the cellular immune system, and

the finding of high levels of IFN-γ and sIL-2R in children with autoimmune hepatitis is consistent with the previous finding that as increased number of circulating activated T lymphocytes expressing the IL-2 receptor is associated with this disorder and with disease activity (Lobo-Yeo et al., 1990B). The finding of high levels of IFN-γ (T_H1 derived) but not IL-4 (T_H2 derived) in children with autoimmune hepatitis in our study indicates that the disease could be mediated by T_H1 and not T_H2 cells. It has been proposed that the balance of T_H1 and T_H2 cells is critical in the development of autoimmune disease, as discussed for Type 1 diabetes (vide 5.7). Results obtained here again lend support to this hypothesis, in that the predominant circulating cytokine profile in patients with autoimmune hepatitis is T_H1, with little evidence of release of T_H2 cytokines.

The mechanism by which an autoimmune reaction is initiated against hepatocytes is not known. However, viral infections could initiate the immune process and are capable of inducing secretion of cytokines such as IFN-γ, TNF-α, IL-1β and IL-6 as discussed previously (vide 5.7).

The finding of higher levels of cytokines in patients with cirrhosis, than in patients without cirrhosis in the present study is consistent with the hypothesis that cytokines are the major regulator of the development of fibrosis development (Castilla *et al*., 1991). The involvement of both IL-1β and TNF-α in fibroblast proliferation has been shown in vitro and has been reported in a variety of fibrotic disorders (Chojkier and Brenner, 1988; Kovacs, 1991). Increased levels of cytokines in a considerable number of patients with fibrosis in the present study, independent of the causative factors, indicates that cytokines may have a role in the fibrotic and cirrhotic transformation of the liver.

The observation that levels of cytokines are higher in patients with active disease, who have severe hepatic inflammatory infiltration and high levels of transaminases (an index of membrane disruption) suggests a possible role of cytokines in causing liver damage directly. The hypothesis that these cytokines are involved directly in damaging the hepatocyte has been proposed. The ability of cytokines to harm a variety of cell targets is documented (Brennan and Feldmann, 1992; Gilles et al., 1992; Shinagawa et al., 1991; Pujol-Borrell et al., 1987). Hepatocytes have been shown not only to be selectively vulnerable to the action of cytokines in an HBsAg-positive transgenic mouse model (Gilles et al., 1992) but also normal hepatocytes in culture undergo cytolysis in the presence of TNF-α and IFN-γ (Shinagawa et al., 1991). Alternatively, cytokines could cause the death of hepatocytes indirectly. IFN-γ for example, induces the expression of class II MHC

products on hepatocytes in vitro (Franco et al., 1988). It has been proposed that aberrant expression of class II MHC molecules might promote presentation of self antigens by the hepatocytes to T-lymphocytes, resulting in the initiation of an autoimmune attack and subsequent hepatocyte death (Rosa and Fellous, 1984; Lobo Yeo et al., 1990A; Senaldi et al., 1991).

In summary, high levels IFN-γ and sIL-2R are found in children with autoimmune hepatitis indicating a predominant T_H1 cytokine profile. The finding of high levels of cytokines in other chronic liver disease could be a consequence of chronic liver failure and impaired cytokine clearance. Elevated levels of cytokines in patients with active disease suggests a pathogenic role in chronic liver disease and cytokines may have a role in fibrotic and cirrhotic transformation of the liver in these patients.

CHAPTER 12

CELLULAR EXPRESSION OF TUMOUR NECROSIS FACTOR-α AND INTERFERON-γ IN LIVER BIOPSIES OF CHILDREN WITH CHRONIC LIVER DISEASE

12.1 SUBJECTS

A total of 36 patients were studied. Twenty-one patients had autoimmune liver disease: eleven had autoimmune hepatitis (**Table 12.1**) and were diagnosed according to internationally agreed criteria (Scheuer, 1991). Ten were females (median age 12, range 2-14 years). Four were positive for ANA and SMA, one for ANA alone and one for SMA alone, while 5 were positive for LKM-1. Six patients were tested before immunosuppressive therapy was started and had high values of AST, IgG, non-organ specific autoantibodies and severe piecemeal necrosis in the liver biopsy. Four were tested while on immunosuppressive treatment (azathioprine 1-1.5 mg/kg/day and/or prednisolone 0.5-2 mg/kg/day). Two of these had severe a portal tract infiltrate in the liver biopsy, one moderate and one mild. Ten children had autoimmune sclerosing cholangitis (**Table 12.1**). Five were females (median age 13, range 4-18 years), all had characteristic changes on endoscopic retrograde cholangio-pancreatography (El-Shabrawi et al., 1987). Seven were positive for SMA and ANA, two for SMA only and one for ANA only. Five were tested at diagnosis before therapy; two of them had mild and three a severe portal tract inflammatory cell infiltrate. Five patients were tested while on treatment with azathioprine (1.5 mg/kg/day) and/or prednisolone (0.5-1.5 mg/kg/day). One of them was also receiving colchicine 25 μg/kg/day. Of these patients, three had mild, one moderate and one a severe portal tract inflammatory cell infiltrate.

As a pathological control, fifteen children with liver biopsies showing a mononuclear cell infiltrate in the portal tract were studied (**Table 12.2**): eight had Wilson's disease (6 male, median age 14, range 3-20 years), one with severe, two with moderate and five with a mild portal tract inflammatory changes. Five were untreated, two were receiving penicillamine 20 mg/kg/day, one trientine 300 mg/daily and zinc sulphate 250 mg/daily. The other seven had alpha-1-antitrypsin deficiency (4 female, median age 5, range 3-14 years), 6 were receiving no treatment while one was receiving colchicine (25 μg/kg/day) (**Table 12.2**). Six of them had mild and one moderate portal tract lymphocytic infiltrate. All patients with Wilson's disease and alpha 1 antitrypsin deficiency were negative for ANA, SMA and LKM autoantibodies.

Four normal liver biopsies obtained from transplant donors were studied as controls (3 male and one female, ages 19, 20, 45 and 26 years).

Table 12.1 Clinical, biochemical, immunological and histological details of patients with autoimmune hepatitis (AIH).

Patients	Sex	Age (yrs)	AAB	AST (IU/l)	IgG (g/l)	Treatment	Portal and peri-portal tract infiltrate*	TNF-α#	IFN-gamma#
AIH									
N.B	F	9	ANA SMA	105	17.9	NO	SEVERE	0	0
S.G	F	15	ANA SMA	601	42.3	NO	SEVERE	2	2
S.W	F	14	LKM-1	74	17.1	NO	SEVERE	2	2
L.A	F	9	LKM-1	105	17.2	NO	SEVERE	1	2
K.E	F	2	LKM-1	2000	21.3	NO	SEVERE	3	3
V.T	F	3	LKM-1	980	21.3	NO	SEVERE	3	3
L.B	F	10	ANA	30	15.2	P	MILD	1	0
J.C	F	14	SMA	465	35.9	P	SEVERE	2	2
B.M	F	6	LKM-1	403	20.6	P	MILD	1	1
H.S	F	14	ANA SMA	43	18.7	P+A	MODERATE	2	2
L.H	M	14	ANA SMA	54	13.9	P+A	SEVERE	2	2

AIH= Autoimmune hepatitis, AAB= Auto-antibody, ANA= Antinuclear antibody, SMA= Smooth muscle antibody, LKM-1= Liver-Kidney microsomal type 1 antibody. AST= Aspartate aminotransferase (normal levels <45 IU/L), IgG= Immunoglobulin G (normal levels <16 g/l). Treatment: P= Prednisolone, A= Azathioprine, NO= no treatment. *Inflammatory cell infiltrate in liver biopsy samples were assessed using a semiquantitative scale (mild, moderate and severe) according to Scheuer (1991). #Score of the expression of TNF-α and IFN-gamma producing cells within the mononuclear cell infiltrate. The grading was: 0= no positive cells, 1 minimal= < 5%, 2 moderate= 5-10% and abundant= >10%.

Table 12.2 Clinical, biochemical, immunological and histological details of patients with autoimmune sclerosing cholangitis.

Patients	Sex	Age (yrs)	AAB	AST (IU/l)	IgG (g/l)	Treatment	Portal and peri-portal tract infiltrate*	TNF-α#	IFN-gamma#
ASC									
M.P	M	15	SMA	24	13.6	NO	MILD	1	2
C.M	M	9	SMA	165	16.4	NO	MILD	0	0
O.S	M	14	ANA SMA	389	29.4	NO	SEVERE	1	1
Z.R	M	4	ANA SMA	174	70.8	NO	SEVERE	0	0
P.T	F	13	ANA	277	16.9	NO	SEVERE	2	2
S.R	F	10	ANA SMA	29	8.8	P	MILD	1	1
C.J	F	10	ANA SMA	150	14.2	P	MILD	0	0
S.S	F	15	ANA SMA	295	51.9	P+A	SEVERE	3	3
H.Y	M	18	ANA SMA	28	13.7	P+A	MILD	1	2
K.C	F	13	ANA SMA	211	15.5	P+A+ COLC	MODERATE	1	2

ASC= Autoimmune sclerosing cholangitis, AAB= Auto-antibody, ANA= Antinuclear antibody, SMA= Smooth muscle antibody. AST= Aspartate aminotransferase (normal levels <45 IU/L), IgG= Immunoglobulin G (normal levels <16 g/l). Treatment: P= Prednisolone, A= Azathioprine, COLC= Colchicine, NO= no treatment. *Inflammatory cell infiltrate in liver biopsy samples were assessed using a semiquantitative scale (mild, moderate and severe) according to Scheuer (1991). #Score of the expression of TNF-α and IFN-gamma producing cells within the mononuclear cell infiltrate. The grading was: 0= no positive cells, 1 minimal= < 5%, 2 moderate= 5-10% and abundant= >10%.

Table 12.3 Clinical, biochemical and histological details of patients with Wilson's disease (WD) and alpha-1-antitrypsin deficiency (A1ATD)

Patients	Sex	Age (yrs)	AAB	AST (IU/l)	IgG (g/l)	Treatment	Portal and peri-portal tract infiltrate*	TNF-α#	IFN-gamma#
WD									
P.B	M	14	neg	57	8.4	NO	MILD	neg	neg
J.R	M	14	neg	88	8.1	NO	SEVERE	1	neg
L.R	F	13	neg	109	9.59	NO	MODERATE	neg	neg
S.R	M	12	neg	180	10.5	NO	MODERATE	neg	neg
S.W	F	3	neg	42	-	NO	MILD	neg	neg
H.W	M	10	neg	132	7.55	Pe	MILD	neg	neg
C.A	M	20	neg	38	14	Pe	MILD	neg	neg
S.H	M	15	neg	35	18.2	T+Z	MILD	neg	neg
A1ATD									
A.C	F	3	neg	50	6.12	NO	MILD	neg	neg
L.C	F	5	neg	132	10.2	NO	MODERATE	neg	neg
L.P	F	5	neg	235	12	NO	MILD	neg	neg
S.P	M	14	neg	44	9.1	NO	MILD	neg	neg
M.W	M	6	neg	57	7.12	NO	MILD	neg	neg
B.W	M	4	neg	79	5.68	NO	MILD	neg	neg
E.D	F	5	neg	27	8.32	COL	MILD	neg	neg

AAB= Auto-antibody. AST= Aspartate aminotransferase (normal levels <45 IU/L), IgG= Immunoglobulin G (normal levels <16 g/l) AST= Aspartate aminotransferase (normal levels <45 IU/L). Treatment; Pe= Penicillamine, T= Trientine, Z= Zinc sulphate, NO= no treatment. *Inflammatory cell infiltrate in liver biopsy samples were assessed using a semiquantitative scale (mild, moderate and severe) according to Scheuer (1991). #Score of the expression of TNF-α and IFN-gamma producing cells within the mononuclear cell infiltrate. The grading was: 0= no positive cells, 1 minimal= <5%, 2 moderate= 5-10% and abundant= >10%.

12.2 RESULTS

Both TNF-α and IFN-γ producing cells were detected within the inflammatory cell infiltrate in the liver biopsies of patients with autoimmune liver disease and showed a diffuse cytoplasmic staining (**Figure 12.1 and Figure 12.2**). Ten of 11 children with autoimmune hepatitis were positive for TNF-α and 9 for IFN-γ. Both cytokines were detected simultaneously in nine children (**Figure 12.3A** and **3B**). Positive staining for both TNF-α and IFN-γ was also present in 7 of 10 patients with autoimmune sclerosing cholangitis. The score of the expression of TNF-α correlated significantly with the score of the expression of IFN-γ ($r=0.62$, $p<0.02$) (**Table 12.1**).

TNF-α staining was positive in only 1 out of 8 patients with WD while IFN-γ was detected in none of them. None of the sections from patients with A1ATD or from the livers of organ donors were positive for either cytokine (**Table 12.2**). The frequency of expression of TNF-α and IFN-γ was significantly higher in patients with autoimmune diseases than in patients with metabolic diseases ($\chi^2= 11$, $p<0.001$ for both cytokines).

The association between the degree of inflammatory cell infiltrate and positive staining for cytokines was also studied. In children with autoimmune liver disease, there was a significant correlation between score of cytokine expression and severity of inflammatory cell infiltrate (for TNF-α, $r=0.50$, $p<0.03$ and for IFN-γ, $r=0.43$, $p<0.05$). The only patient amongst those with metabolic diseases in whom a minimal number of TNF-α stained cells was detected, a child with Wilson's disease, had severe portal tract inflammatory changes. In children with autoimmune liver disease, there was a significant correlation between the score of both cytokine expression and levels of AST ($r=0.56$, $p<0.008$ for TNF-α and $r=0.47$, $p<0.03$ for IFN-γ) (**Figure 12.4**). No correlation was observed between the levels of IgG and the score of cytokine expression. The score of expression of TNF-α and IFN-γ was not different between treated and untreated patients. In autoimmune liver disease, when there were <10% of cells positive for either cytokine, there were typically within portal tract. In contrast, when >10% of cells were positive, they were typically seen within both portal and peri-portal areas (**Figure 12.2**).

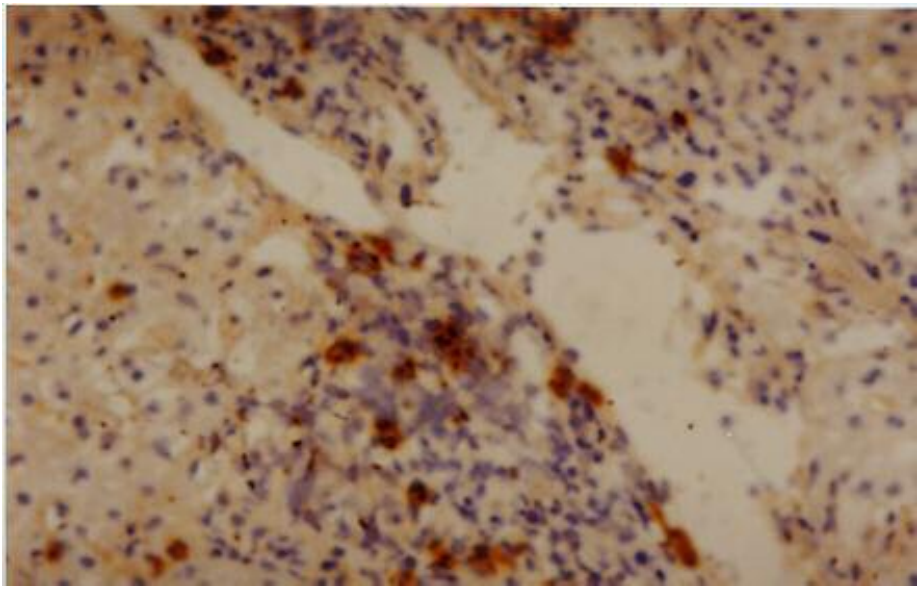


Figure 12.1 Liver section from a patient with autoimmune hepatitis showing large cells with a diffuse cytoplasmic staining for TNF-α within the portal inflammatory cell infiltrate. (Immunoperoxidase staining, original magnification 40x)

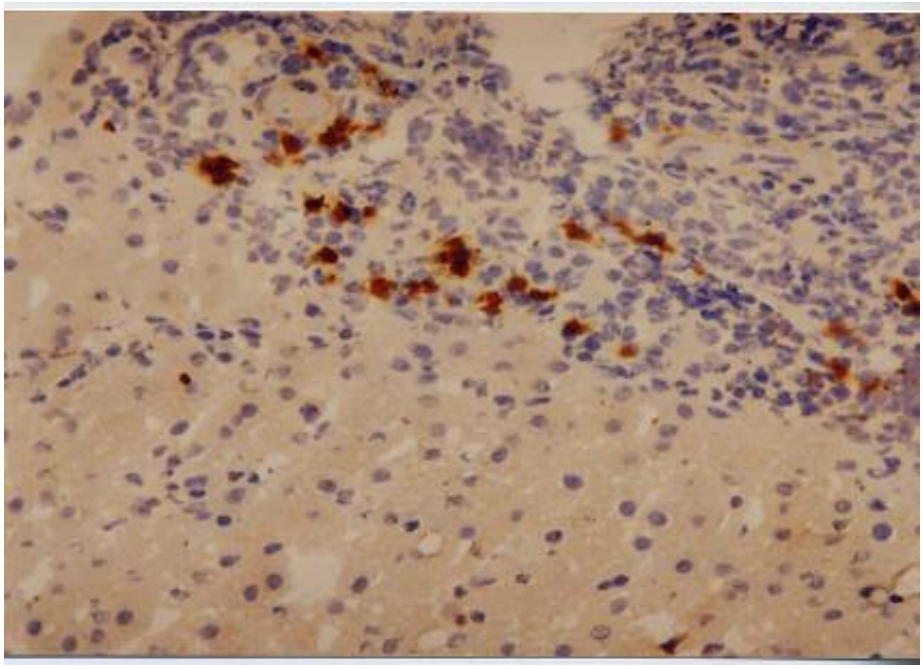
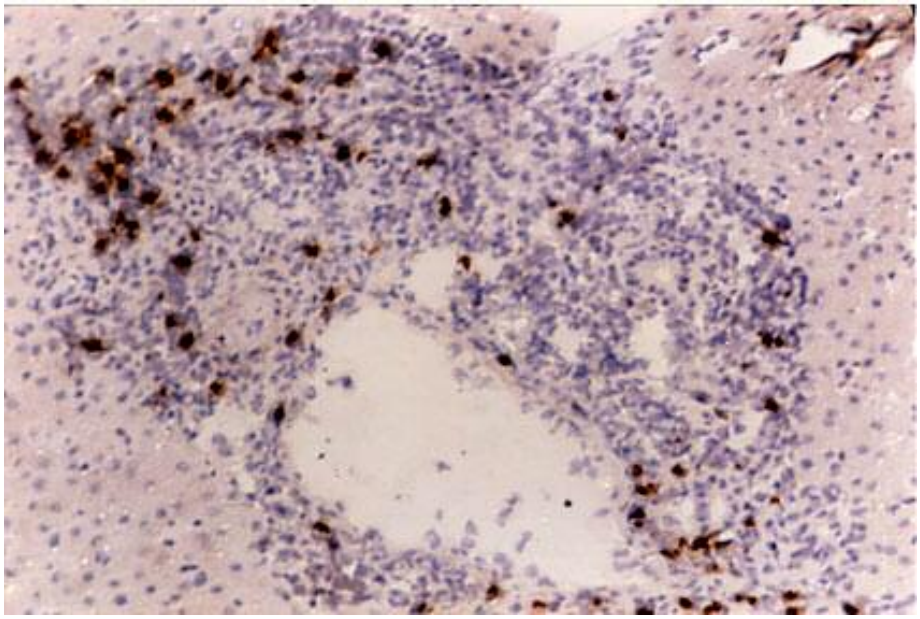
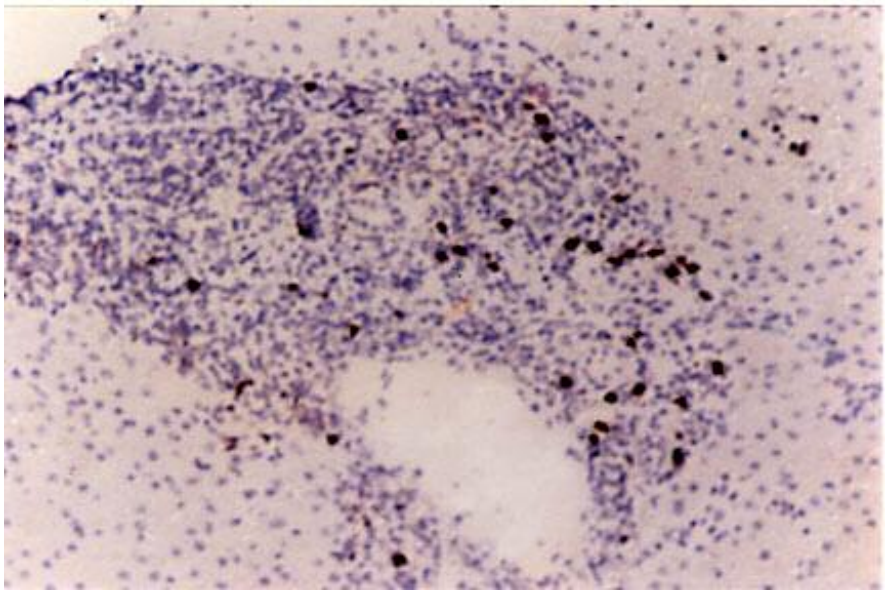


Figure 12.2 Liver section from a patient with autoimmune sclerosing cholangitis showing large cells with a diffuse cytoplasmic staining of IFN-γ within the inflammatory cell infiltrate. Positively stained cells are mainly within the portal tract, with a few occurring some distance into the parenchyma. (Immunoperoxidase staining, original magnification 25x)



(A)



(B)

Figure 12.3. Liver sections from a patient with autoimmune hepatitis. Simultaneous presence of cells positively stained for TNF-α (3A) and IFN-γ (3B) within the portal inflammatory cell infiltrate. (Immunoperoxidase staining, original magnification 25x).

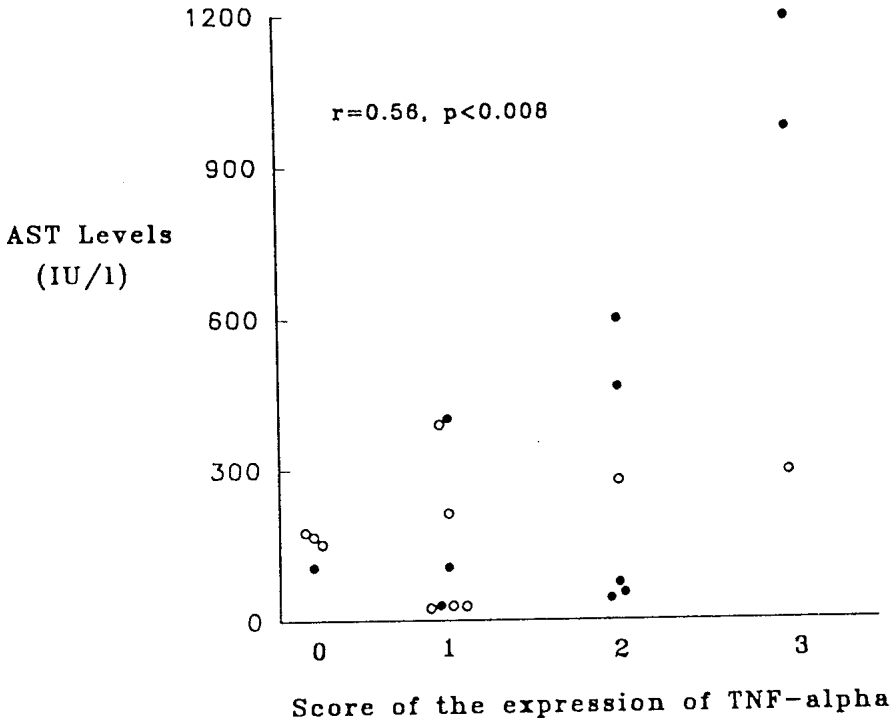


Figure 12.4. Correlation between levels of aspartate aminotransferase (AST) and the score of the expression of TNF-α in children with autoimmune liver disease (●=patients with autoimmune hepatitis, O=patients with autoimmune sclerosing cholangitis).

12.3 COMMENT

The results in this section show that cells producing TNF-α and IFN-γ are present in the liver inflammatory infiltrate of children with autoimmune chronic liver diseases but are rarely seen in metabolic diseases such as Wilson's disease and alpha 1 antitrypsin deficiency. This suggests that cytokines may play a role in autoimmune liver damage. The observation that the number of cytokine-producing cells correlates with both levels of transaminases and the magnitude of the inflammatory infiltrate suggests potential mechanisms whereby autoimmune liver damage arises.

A direct cytolytic mechanism is suggested by the relationship between the number of cytokine-producing cells and increased circulating levels of the transaminase AST. In this scenario, the well-known ability of TNF-α and IFN-γ, both independently and synergistically, to damage a variety of cell targets inducing

hepatocytes may account for autoimmune liver damage, as discussed previously (vide 11.3).

Cytokine-mediated recruitment of inflammatory cells to the inflamed area is another possible mode of liver cell injury in autoimmune liver disease. This mechanism is suggested by the correlation between the number of cytokine-producing cells and the overall severity of the mononuclear cell infiltrate. Both TNF-α and IFN-γ have chemotactic properties and are thought to increase the cellular traffic into sites of inflammation and to enhance the immune response by increasing the co-stimulatory signals required for T cell activation (Munro et al., 1989; Thornhill et al., 1991). Although some of the children with metabolic diseases studied had inflammatory cell infiltrates of a severity similar to those with autoimmune disease, they did not have cells positively staining for TNF-α and IFN-γ, suggesting that these two cytokines are closely linked to the autoimmune nature of the target organ damage.

The fact that the presence of TNF-α producing-cells strongly correlates with that of IFN-γ producing-cells suggests that these two cell types have interacting functions. Since IFN-γ producing-cells typically belong to the T_H1 subset, it is possible to suggest the following pathogenic scenario. Recirculating T_H1 cells encounter their specific antigenic peptide in the liver, presented by HLA class II on professional antigen presenting cells such as the Kupffer cells. Recognition of the antigen leads T_H1 cells to release the powerful macrophage activator, IFN-γ. This in turn leads cells of the macrophage lineage to release the pro-inflammatory cytokine TNF-α. The two cytokines can thus deliver their pathogenic potential by i) enhancing/inducing HLA class II molecules, a critical step in the process of antigen recognition, ii) recruiting inflammatory cells to the site of the lesion, iii) exerting a direct cytotoxic effect on the hepatocyte. In any of these cases, the nature of the initial stimulus, the antigenic peptide, remains to be elucidated.

The view that cytokine producing cells are involved in the pathogenic process of autoimmune liver disease is supported by observations that in other autoimmune diseases such as Type 1 diabetes and rheumatoid arthritis, TNF-α and IFN-γ producing-cells are prominent in the characteristic lesions, namely the insulitis and the inflamed synovium respectively (Foulis et al., 1991; Chu et al., 1991).

In conclusion, the relationship between numbers of cytokine producing cells, the severity of the inflammatory infiltrate and the levels of transaminases suggests a pathogenic role for cytokines in autoimmune liver disease. If this hypothesis is

correct, anti-cytokine antibody treatment (Shinagawa et al., 1991) which has been effective in animal models of cytokine-induced liver damage, or the use of cytokine antagonists (Dinarello et al., 1991) would be worth considering in the treatment of autoimmune liver disease.

CHAPTER 13

GENERAL DISCUSSION

GENERAL DISCUSSION

13.1 ONSET OF TYPE 1 DIABETES IS ASSOCIATED WITH PRODUCTION OF T_H1 CYTOKINES

The main objective of this thesis was to assess the involvement of cytokines in the development of Type 1 diabetes using a predominantly *in vivo* approach. Cytokines have previously been implicated in the immunopathology of several autoimmune diseases including Type 1 diabetes (Dinarello and Mier, 1987). There is evidence from *in vitro* studies that cytokines could have a direct role in promoting β-cell death (Mandrup-Poulsen *et al*., 1989). However, evidence from *in vivo* studies to support a role for cytokines in the pathogenesis of Type 1 diabetes is scant. The results in this thesis indicate that serum levels of four of the eight cytokines measured - namely IL-2, IFN-γ, TNF-α and IL-1α - are increased in recently diagnosed patients with Type 1 diabetes when compared to both disease and metabolic control subjects and also to normal controls. At diagnosis of Type 1 diabetes, 89% of patients had elevated levels of at least one of these four cytokines. This finding strongly suggests that T_H1 cells are activated in these patients, leading in turn to enhanced production of macrophage-derived cytokines such as TNF-α and IL-1α.

There are a number of possible alternative reasons why circulating cytokine levels may be elevated in recently diagnosed Type 1 diabetes. Concomitant illness such as infection may be associated with raised levels of cytokines in patients at diagnosis of diabetes but cannot explain the elevated levels in long-standing patients who were without clinical symptoms of illness at the time of sampling. Metabolic disturbance or chronic autoimmunity are unlikely to result in these findings since levels of cytokines did not correlate with patients' blood glucose levels or the presence of antibodies to ICA or GAD. It is likely therefore that the high levels of cytokines noted in this thesis are related to an immune process associated with Type 1 diabetes of recent onset. This immune process is probably relevant to the development of disease since elevated levels of activated T cells have been repeatedly demonstrated in recently diagnosed patients with Type 1 diabetes (Alviggi *et al*, 1984; Peakman *et al*, 1990) and also in prediabetic twins but not in twins who did not develop Type 1 diabetes (Tun *et al*.,

1994).

It has previously been proposed that the balance between T_H1 and T_H2 cells is critical in the development of Type 1 diabetes (Harrison *et al*., 1993). This suggestion is made on the basis that at-risk first degree relatives who have poor delayed-type hypersensitivity response to specific islet antigens (eg. GAD) may be less likely to develop diabetes (Harrison *et al*., 1993). In addition, there is evidence from the non-obese diabetic (NOD) mouse model of spontaneous diabetes that GAD-responsive, IFN-γ producing T lymphocytes are an early feature of the disease process (Kaufman *et al*., 1993). The findings in this thesis complement these other reports by providing evidence that T_H1 cell activation is found in the majority of patients with Type 1 diabetes. By contrast, the finding of elevated levels of T_H2 cytokines (IL-4 and IL-10) in patients with Graves' disease is of interest since it is consistent with an antibody-mediated pathogenesis for this disease.

The mechanism by which autoimmune reactions are initiated against the β-cells and the stimulus for cytokine production in Type 1 diabetes are not known. Viral infection could initiate the immune process since viruses are capable of inducing IFN-γ (Trinchieri and Perussia, 1985) and have also been shown to induce secretion of other cytokines such as TNF-α, IL-1β and IL-6 from human monocytes (Henke *et al*., 1992).

The results obtained are in agreement with Cavallo *et al*. (1991) who found that levels of TNF-α are elevated in a proportion of recently diagnosed patients with Type 1 diabetes. Elevated levels of IFN-γ in recently diagnosed patients with Type 1 diabetes in the present study is concordant with the observation of Tova *et al*. (1984) but is in contrast with the study of Cavallo *et al*. (1991) who found that levels of IL-1α and IFN-γ in patients with Type 1 diabetes were normal. Methodological differences such as differences in assay condition are likely to account for this discrepancy. Furthermore, since cytokines are labile molecules with short half-lives, serum samples used in this study were stored at -70^{o}C and not at -20^{o}C as in that study (Cavallo *et al*. 1991). Mooradian *et al*. (1991) found no detectable levels of IL-1α and IL-1β in a group of long-standing patients with hyperglycaemia, although the nature of diabetes (Type 1 or Type 2) in these

patients was not stated. In the present study IL-1β was not detected in serum, possibly due to the inability of our assay to measure IL-1β in serum, the presence of cytokine inhibitors such as IL-1β type II receptors which are naturally occurring inhibitors for IL-1β, but do not bind IL-1α (Scott *et al*., 1991). Elevated levels of IL-4, IL-6 and IL-10 were not detectable in the serum of any of the diabetic or normal control subjects in this thesis, in agreement with the finding of Cavallo *et al*. (1991) that levels of IL-4 and IL-6 are undetectable in patients with Type 1 diabetes, indicating a lack of recruitment of the T_H2 subset at diagnosis of Type 1 diabetes.

In summary, this study shows that high levels of circulating IL-2,-IFN-γ, IL-1α and TNF-α are associated with the diagnosis of Type 1 diabetes. This supports the recent proposal that preferential activation of T_H1 lymphocytes is a key element in the development of Type 1 diabetes (Harrison *et al*., 1993). In order to determine whether T_H1 activation precedes the clinical onset of Type 1 diabetes, circulating cytokine profiles were characterised in a cohort of-non-diabetic identical twins of patients with Type 1 diabetes, some of whom went on to develop the disease themselves.

13.2 CYTOKINE PROFILE IN A COHORT OF NON-DIABETIC IDENTICAL TWINS OF PATIENTS WITH TYPE 1 DIABETES

The investigation was then extended to twins to determine whether abnormally elevated levels of cytokines is similar in prediabetic and non-diabetic twins who have a relatively high risk of developing the disease themselves. Levels of macrophage-derived cytokines (TNF-α and IL-1α) were elevated above the normal range with a higher prevalence in the twins who developed diabetes than in those who did not. These findings are consistent with evidence that the appearance of macrophages in islets of Langerhans is the first event heralding the onset of insulitis in the NOD mouse (Walker *et al*., 1988). In addition, manoeuvres designed to inhibit macrophage function, such as administration of silica, inhibit the development of diabetes in the BB rat (Oschilewski *et al*., 1985).

The analysis of the T_H1 and T_H2 cytokine profiles did not reveal a clear difference in the pattern obtained in the prediabetic twins compared with the twins

remaining non-diabetic although some prediabetic twins had primarily a T_H1 profile of circulating cytokines. However, levels of cytokines typical of a T_H2 profile were higher in non-diabetic twins than in patients with recently diagnosed Type 1 diabetes. This raises the possibility that T_H2 cytokines could protect against onset of Type 1 diabetes, perhaps reflecting an "appropriate" immune response to environmental trigger(s) which initiate the autoimmune process. This concept is supported by a number of lines of evidence. Firstly, administration of IL-4 (Rapoport *et al*., 1993; Fowell and Mason, 1993) or IL-10 (Pennline *et al*., 1994) to NOD mice prevents the onset of diabetes in these animals. In addition, the adoptive transfer of IL-4-secreting T_H2 splenocytes into neonatal NOD mice prevents the progression of diabetes while transfer of IFN-γ-secreting T_H1 cells promotes the onset of disease (Katz *et al*., 1995; Healy *et al*., 1995).

The mechanism by which T_H2 cells protect against the development of Type 1 diabetes is unknown; however, a possible role for T_H2 cytokines (IL-4 and IL-10) in preventing the disease processes by down-regulating autoimmune responsiveness has been suggested. This is supported by evidence suggesting that T_H1 and T_H2 subsets are mutually antagonistic. For example, IL-10 suppresses synthesis of T_H1 helper cytokines such as IL-2 and IFN-γ (Moore *et al*., 1993). It has also been shown that IL-4 suppresses LPS-induced stimulation of IL-1, TNF-α and IL-6 synthesis by human monocytes (Cheung *et al*., 1990). These observations are in keeping with the finding in the present study that high levels of IL-4 and IL-10 in non-diabetic twins are associated with low levels of TNF-α and IL-1α.

In summary, this study has shown that macrophage-derived cytokines are elevated to a greater extent in twins who progress to diabetes than in those who do not. While the ratio of T_H1/T_H2 cytokines was not correlated with the disease progression, non-diabetic twins exhibited higher levels of T_H2 cytokines than newly diagnosed diabetes. In order to understand the factors which influence cytokines production in diabetic patients, the role of genetic traits in this process was examined.

13.3.1 THE ROLE OF UNDERLYING GENETIC TRAITS IN INFLUENCING CYTOKINE LEVELS IN PATIENTS WITH TYPE 1

DIABETES AND THEIR SIBLINGS AND PARENTS

To determine whether cytokines levels are genetically determined, measurements were made of circulating cytokine levels and mitogen-stimulated secretion of cytokines by PBMC in both diabetic children and their first degree relatives. These results indicated that circulating levels of TNF-α and sIL-2R are elevated in non-diabetic first degree relatives (i.e. sibs and parents), as well as in children with Type 1 diabetes. In addition, hypersecretion of IL-1α and TNF-α by mitogen-stimulated PBMC was found in this population. These abnormalities of cytokine production in first degree relatives are of interest since these individuals have a relatively low risk of progression to diabetes (Allen *et al*., 1991). Thus, overproduction of IL-1α and TNF-α is not sufficient in isolation to promote diabetes.

One possible explanation for the increased circulating levels of cytokines and their hypersecretion by PBMC in the first degree relatives is that the immune system is hyperactive in these individuals. Overactivity of the immune system has been implicated as a possible predisposing factor to autoimmune diseases in individuals with particular HLA class II alleles-(HLA-DR3/DR4) (Jaworski *et al*., 1983). However in the present study, no association between HLA DR3/4 and either circulating levels of cytokines nor their secretion by PBMC was found in both diabetic children or their relatives, suggesting that the DR3/4 alleles do not exercise a unequivocal influence on determining high cytokine levels. This finding is in agreement with the study by Mølvig *et al.* (1990) who did not find a significant association between production of IL-1β and TNF-α by monocytes and HLA-DR phenotype.

It is possible that other alleles could be linked to the abnormalities in cytokine secretion reported here. The genes encoding TNF-α and TNF-β are both located on the short arm of chromosome 6 between HLA B and HLA DR loci (Campbell and Trowsdale, 1993). Wilson *et al*. (1993) have shown that a polymorphism in the TNF-α gene is strongly associated with the HLA A1, B8, DR3 haplotype, which is prevalent in Type 1 diabetes and in other autoimmune diseases. Furthermore, RFLP analysis of TNF-β gene has shown that fragments of 5.5 kb and 10.5 kb are associated with Type 1 diabetes susceptibility haplotypes

(DR3/4) (Pociot *et al*., 1991) and are commonly found in both diabetics and in their first degree relatives (Ilonen *et al*., 1992). Conseqently, it would be interesting to examine whether the elevated production of TNF-α found in diabetic children and their relatives is associated with any of the TNF-gene polymorphisms described in these studies.

The data presented in this thesis suggest that genetic factors may influence cytokine levels in the diabetic children and in first degree relatives. However, two pieces of evidence indicate that genetic background is not the sole influence on circulating TNF-α levels in these subjects. Firstly, although elevated, TNF-α levels were lower in first degree relatives than in either prediabetic twins or recently diagnosed patients with Type 1 diabetes. In addition, TNF-α levels were similar in non-diabetic twins compared to normal controls. Thus, environmental factors, such as viral infection, must also contribute to enhanced TNF-α production in diabetic patients.

To investigate the activation of macrophages and T cells in diabetic children, cytokine secretion by mitogen stimulated PMBC was measured.

13.3.2 MITOGEN-STIMULATED SECRETION OF CYTOKINES BY PBMC IN IN CHILDREN WITH TYPE 1 DIABETES

In support of a central role for macrophages in the pathogenesis of Type 1 diabetes, the results in the present study demonstrate that LPS-stimulated IL-1α and TNF-α secretion by PBMC *in vitro* is significantly increased in diabetic children. In support of this, hypersecretion of TNF-α has previously been reported in an animal model of diabetes (Rothe *et al*., 1990). That study indicates that following activation by LPS, peritoneal macrophages from diabetes-prone BB rats secrete strikingly higher amounts of TNF-α than those from diabetes-resistant or normal Wistar rats. However, these findings contrast with an earlier study in the NOD mouse by Satoh *et al*. who found that the induction of TNF-α *in vitro* after LPS stimulation was lower than in control mice (Satoh *et al*., 1989) and with studies showing no alteration in TNF-α secretion (Mølviq *et al*., 1990; Mooradian *et al*., 1991; Ohno *et al*., 1993) compared with normal animals. Discrepancy between the results presented here and by others could be explained by different

experimental conditions employed such as the number of cells and concentration of mitogen used in assays (Ohno *et al*., 1993; Mølviq *et al*., 1990). In addition, in one study the nature of the diabetes in those patients was not given (Mooradian *et al*., 1991).

Production of cytokines such as IL-2 and IFN-γ by mitogen (PHA) stimulated-T cells has been reported in patients with Type 1 diabetes. Toms *et al.* (1991) found normal production of IFN-γ, concordant with the results in this thesis, suggesting that T cell function in Type 1 diabetes is not impaired although the present findings are in contrast with earlier studies by Kaye *et al*., (1986) and Giordano *et al*., (1989) who found a decreased synthesis of IL-2 by mitogen-stimulated lymphocytes from patients with Type 1 diabetes. Again these differences may be explained by differences in assay conditions such as concentration and duration of exposure to mitogen.

The finding of elevated levels of TNF-α and sIL-2R in diabetic children is in agreement with the data presented here (section **5.2)** indicating that these cytokines are also elevated in patients of with recently diagnosed Type 1 diabetes. These findings indicate that activation of macrophages and T cells is closely linked to Type 1 diabetes (Foulis *et al*., 1991; Lee *et al*., 1988). Levels IL-1α and IFN-γ in diabetic children are not significantly different when compared with normal controls. The results observed are in agreement with the previous studies by Mooradian *et al*. (1991) who found that levels of IL-1α and IL-1ß in a group of long-standing patients with hyperglycaemia were not different when compared with normal subjects. By contrast, Tova *et al*. (1984) found elevated levels of IFN-γ in recently diagnosed Type 1 diabetes. This difference could be explained by the duration of the disease in the patients studied.

In summary, elevated circulating levels of TNF-α and sIL-2R and hypersecretion of macrophage-derived cytokines by PBMC was found in diabetic children. This supports the hypothesis that activation of macrophages and T cells is associated with Type 1 diabetes although is highly unlikely in isolation to explain the pathogenesis of this disease. Elevated levels of cytokines (circulating and secreted by PBMC) are also present in first degree relatives of children with Type 1 diabetes suggesting that the immune system in these individuals is

hyperactive.

While the data presented in this thesis show that abnormalities in cytokine levels are associated with Type 1 diabetes, they do not address the issue of local cytokine production within the pancreas. In order to examine this question, cytokines producing cells were examined in the closest animal model of human-insulin-dependent diabetes, the NOD mouse.

13.4 PRODUCTION OF CIRCULATING AND PANCREATIC CYTOKINES IN AN ANIMAL MODEL OF DIABETES - THE NOD MOUSE

Cytokines were studied in the NOD mouse, an animal model bearing a striking resemblance to human Type 1 diabetes. In this setting, it was possible to investigate cytokines in the circulation and in the pancreas and to address the question, unanswerable in humans, as whether the events occurring in the islets are reflected in the circulation.

Similar to patients with Type 1 diabetes (**chapter 5.2**), circulating levels of cytokines produced by macrophages (TNF-α) and T_H1 cells (IL-2) were elevated in association with the development of diabetes in this animal model of Type 1 diabetes. Furthermore, in agreement with the human data presented earlier (**chapter 5.2**), elevated levels of IL-4 (T_H2 cytokine) were not detectable in the serum of either test or controls mice. The association between overproduction of T_H1 cytokines and onset of diabetes in the NOD mouse is in agreement with a number of other studies. Katz *et al*., 1995 have demonstrated that diabetes is rapidly induced in neonatal NOD mice after transfer of T_H1-like cells in almost all recipients while none of recipients of T_H2-like cells developed diabetes. The generation of T_H1 and T_H2 subsets is influenced by the cytokines present during the initial phase of the immune response and a major role is played by IFN-γ and IL-4 (Paul and Seder, 1994). IFN-γ appears to be important in the development of insulin-dependent diabetes as demonstrated by the possibility to induce diabetes in transgenic mice expressing IFN-γ under the control of the insulin promotor (Sarvetuick *et al*., 1988). Conversely, the T_H2-derived cytokines IL-4 and IL-10 appear to inhibit progression to diabetes in NOD mice (Rapoport *et al*., 1993;

Pennline *et al*., 1994). These findings are likely to be of direct relevance to human insulitis in view of the data presented here which indicate the similarity between circulating cytokine levels in NOD mice and in humans with Type 1 diabetes of recent onset. It is unlikely that high levels of cytokines found in NOD mice in the present study are solely determined by genetic factors since levels of IL-2 and TNF-α in non-diabetic NOD mice or NODxB10 F1 mice were within the normal range. Thus, high levels of cytokine production in NOD mice are likely to be related to an immune process triggered by environmental factors (Trinchieri and Perussia, 1985)

The release of TNF-α by cells infiltrating the pancreatic islets is believed to be a major effector mechanism of tissue destruction in Type 1 diabetes (Bendtzen, 1989). Immunohistochemical or immunofluorescence techniques in the present study succeeded in detecting both IFN-γ (T_H1 profile) and TNF-α producing cells in the islet of Langerhans of diabetic NOD mice. The presence of IFN-γ producing cells in the islet of Langerhans of diabetic NOD mice (Rothe *et al*., 1994) and in diabetic patients (Foulis *et al*., 1991) suggests that IFN-γ may also be important in the pathogenesis of this disease. In support of this, it has been reported that IFN-γ upregulates MHC class I and class II antigens on various cells including pancreatic β-cells (Wright *et al*., 1986; Campbell *et al*., 1986) and enhances TNF-induced cytotoxicity of human islet cells (Soldevila *et al*., 1991). In addition, antibodies to IFN-γ protect against diabetes development in NOD mice and BB rats (Debraysachs *et al*., 1991). In the present study, the finding of positive staining for TNF-α apparently within the islet cells gives support to the hypothesis that β-cells themselves are also able to produce cytokines. This concept is supported by *in vitro* experiments showing that pancreatic β-cells produce TNF-α in culture. Furthermore, it has been reported that TNF-α mRNA expression was induced in β-cells after exposure to IL-1 and IFN-γ (Yamada *et al*., 1993) or IL-6 (Campbell *et al*., 1989). These findings suggest that TNF-α produced by islet β-cells may act as a costimulator for autoreactive B and T lymphocytes in autoimmune diabetes. The presence of both TNF-α and IFN-γ producing-cells in pancreatic islets suggests that these two cell types may act in concert to induce MHC class II molecules upon β-cells, a critical step for autoimmune β-cell

destruction. Evidence to support this hypothesis comes from an immunohistochemical study of pancreas obtained from patients with recent onset of diabetes (Foulis *et al*., 1991). This analysis indicated that at diagnosis of diabetes, the islet of Langerhans are infiltrated by mononuclear cells (insulitis), consisting of mainly activated T cells and macrophages. In this study, 40% of the infiltrating lymphocytes stained with antibody to IFN-γ, while the β-cells stained positively for MHC class II expression. Thus, release of IFN-γ by activated T lymphocytes could lead to β-cell damage through induction of TNF release from macrophages (Fowell and Mason, 1993).

In summary, these results demonstrate that, similar to human Type 1 diabetes, circulating levels of cytokines produced by macrophages (TNF-α) and T_H1 cells (IL-2) are elevated in association with the development of diabetes in this animal model and reflect events occurring in the islets. The presence of IFN-γ and TNF-α producing cells in the inflamed islets in diabetic NOD mice but not in animals without the disease implies that these cytokines may be involved in the pathogenesis of Type 1 diabetes.

The data presented here provide strong evidence supporting a role for locally produced cytokines in the development of Type 1 diabetes. In order to examine whether cytokine production contributes to the genesis of other organ-specific autoimmune disease, this study was extended to an examination of cytokine production in auotimmune hepatitis.

13.5 ROLE OF CYTOKINES IN THE PATHOGENESIS OF AUTOIMMUNE HEPATITIS

The role of cytokines in the pathogenesis of other organ-specific autoimmune disease such as autoimmune hepatitis was also investigated, using as controls patients with liver disorders due to metabolic causes (Wilson's disease and alpha-1-antitrypsin deficiency) at various disease stages. Elevated levels of circulating cytokines such as IL-1α, IL-6 and TNF-α were detected in patients with various forms of chronic liver disease, including those with autoimmune hepatitis, when compared with normal controls, consistent with earlier studies by Devière *et al*. (1990), Khoruts *et al*. (1991), Bird *et al*., (1990), and Tilg *et al*. (1992). Levels

of cytokines and-sIL-2R were significantly higher in patients with cirrhosis and active liver disease (severe hepatic inflammation and high levels of transaminases) in agreement with Devière *et al*. (1990) and Tilg *et al*. (1992). The finding of high levels of IFN-γ and sIL-2R in children with autoimmune hepatitis is consistent with the previous finding that increased number of circulating activated T lymphocytes expressing the IL-2 receptor are found in this disorder, particularly in the presence of active disease (Lobo-Yeo *et al*., 1990B). The observation in the present study of high levels of IFN-γ (T_H1 cell-derived) but not IL-4 (T_H2 cell-derived) in children with autoimmune hepatitis suggests that T_H1 cytokines are predominant in this disease.

The fact that cytokine levels are higher in patients with active liver disease raised the possibility that cytokines may cause liver damage directly. In agreement with this, hepatocytes have been shown to be selectively vulnerable to the toxic action of cytokines in an HBsAg-positive transgenic mouse model (Gilles *et al*., 1992). In addition, normal hepatocytes in culture undergo cytolysis in the presence of TNF-α and IFN-γ (Shinagawa *et al*., 1991). An indirect mechanism by which cytokines could cause hepatocyte death is also possible since IFN-γ can induce the expression of class II HLA molecules on hepatocytes *in vitro* (Franco *et al*., 1988; Senaldi *et al*., 1991). This would be expected to promote presentation of self antigens by the hepatocytes to autoreactive T-lymphocytes, resulting in the initiation of an autoimmune attack and subsequent hepatocyte death (Rosa and Fellous, 1984; Lobo Yeo *et al*., 1990A; Senaldi *et al*., 1991).

Levels of cytokines were higher in patients with cirrhosis than in patients without cirrhosis in the present study, a finding consistent with studies which suggest that cytokines are the major regulator of fibrosis development (Kovacs, 1991; Castilla *et al*., 1991). These groups have demonstrated that, in patients with chronic hepatitis, the level of TGFβ1 expression was closely correlated the expression of procollagen and with fibrosis. Furthermore, IL-6 and IL-1 induce acute phase reactants including fibrinogen and also induce proliferation of fibroblasts.

Increased levels of cytokines in chronic liver diseases could be the consequence of different mechanisms. Firstly, following injury the liver shows an

immense ability for regeneration which is cytokine-led (Andus *et al*., 1991). Secondly, it is possible that cytokine clearance is impaired as a result of chronic liver failure (Klapproth *et al*., 1989; Metcalf and Nicola, 1988; Castell *et al*., 1988; Sonne *et al*., 1990). These studies demonstrated the accumulation of TNF, IL-6 and macrophage-colony stimulating factor (M-CSF) in the liver 5-18 minutes after intravenous administration of these cytokines. Thirdly, activation of the cellular immune system may lead to increased serum cytokine levels. This is consistent with the previous finding that an increased number of circulating activated T lymphocytes expressing the IL-2 receptor is associated with these disorders and with disease activity (Lobo-Yeo *et al*., 1990B).

The liver biopsies of patients with autoimmune liver diseases are characterised by a dense mononuclear cell infiltrate in portal tracts. To investigate whether these cells produce TNF-α and IFN-γ, immunohistochemical examination was performed, using anti-TNF-α and IFN-γ Mab. Both TNF-α and IFN-γ producing cells were detected simultaneously within the inflammatory cell infiltrate in the liver biopsies of these patients. Although some of the children with metabolic diseases studied had inflammatory cell infiltrates of a severity similar to those with autoimmune disease, they did not have cells which stained positively for TNF-α and IFN-γ, suggesting that these two cytokines are closely linked to the autoimmune nature of the target organ damage. A direct cytolytic mechanism for these cytokines was suggested by the relationship between the number of cytokine-producing cells found and circulating levels of liver transaminases. This is supported by previous reports which suggest that cytokines can promote hepatocyte death through a direct mechanism (Gilles *et al*., 1992; Shinagawa *et al*., 1991). It is possible, however, that cytokines-induced toxicity may also be mediated through an indirect mechanism such as induction of expression of class II HLA molecules on hepatocytes. This could result in the initiation of an autoimmune attack leading to subsequent hepatocyte death (Rosa and Fellous, 1984; Lobo-Yeo *et al*., 1990A; Senaldi *et al*., 1991).

The concept that cytokine producing cells are involved in the pathogenic process of autoimmune liver disease is supported by the observation that in other autoimmune diseases such as Type 1 diabetes and rheumatoid arthritis, TNF-α and

IFN-γ producing cells are prominent in the characteristic lesions of these two disorders, the insulitic lesion and the inflamed synovium respectively (Foulis *et al*., 1991; Chu *et al*., 1991). However, as is the case for a number of other organ-specific autoimmune diseases, the nature of the initial stimulus and the target antigens involved in the disease process remain unknown.

13.6 PRACTICAL APPROACHES TOWARD THERAPY

The results in this thesis suggest that cytokines are important in the pathogenesis of human Type 1 diabetes and of autoimmune hepatitis. Consequently it would be anticipated that treatment with either anti-cytokine (in particular anti-T_H1 and macrophage-derived cytokines) antibody or T_H2 cytokines (such as IL-4 and IL-10) would be beneficial for the treatments of these autoimmune disease. Recently, it has been suggested that the cellular effector mechanism by which cytokine-induced β-cell death occurs involves the enzyme nitric oxide (NO) synthase, which catalyses the formation of the free radical NO (Corbett *et al*., 1992). This hypothesis proposes that cytokines promote expression of an inducible isoform of NO synthase in the β-cells of pancreatic islets and that causes islet damage (Corbett *et al*., 1991; Rothe *et al*., 1994). Anti-cytokine antibodies and inhibitors of the inducible NO synthase, such as aminoguanidine (Corbett *et al*., 1992) also present a possible therapeutic approach to halt or prevent the diabetogenic process and this study is currently in progress in our laboratory, using the NOD mouse.

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