STUDIES ON THE IMMUNOPATHOGENESIS OF INSULIN-DEPENDENT DIABETES MELLITUS INDUCED IN THE RAT BY THYMECTOMY AND IRRADIATION

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B.Sc. (Hons.)

'This thesis is presented for the degree of Doctor of Philosophy of Murdoch University'
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I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary educational institution.

Philip A Stumbles
ABSTRACT

This study describes the immunological and pathophysiological features of a diabetic syndrome induced in inbred rats by a combination of thymectomy and irradiation (Tx-X), a procedure known to alter immune function and regulation.

The syndrome was characterized clinically by hyperglycaemia, insulinopaenia, ketoacidosis and polyuria. Onset of the disease was rapid and fatal unless daily insulin therapy was initiated immediately. Acutely diabetic animals had pancreatic lesions largely confined to the islets of Langerhans, where atrophy was typically observed. Immunohistochemical studies clearly demonstrated that the atrophic change was due to selective destruction of the $\beta$ cells of the islets, leading to collapse of these structures. Insulitis was also observed in some diabetic rats, particularly those with less acute disease and also occasionally in non-diabetic Tx-X animals, indicating that a sub-clinical form of the disease may occur. Acute diabetic animals developed autoantibodies to islet cells as detected by immunofluorescence, and to a 64kDa islet protein as detected by immunoprecipitation. A large proportion of Tx-X animals also developed a lymphocytic infiltration of thyroid glands and autoantibodies to thyroid components.

The incidence of diabetes ranged from 11 to 62 percent (mean 34 percent). Incidence levels were slightly elevated in males and induction of disease was strain related, but did not depend upon the $RT1^u$ MHC haplotype as does the spontaneous diabetes of BB rats. Incidence and onset of the disease were variable between groups of animals and could be modulated by altering the hygienic background of the animal,
implicating a role for environmental factor(s) in disease pathogenesis. Inhibition of disease by reconstituting Tx-X rats with normal lymphocytes, transfer of disease to naive recipients using diabetic lymphoid cells and demonstration of abnormalities in cell mediated and humoral immunity provided strong evidence supporting an autoimmune pathogenesis for this syndrome.

In conclusion, the Tx-X-induced diabetic syndrome in rats has been shown to bear many features of the human diabetic condition (IDDM) and thus provides further evidence supporting a role for autoimmune mechanisms in the pathogenesis of this major human disease. Finally, it is proposed that this model should provide a valuable adjunct to existing animal models for studying factors influencing the development of this disease and in assessing potential prophylactic or therapeutic regimes.
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CHAPTER 1.0
GENERAL INTRODUCTION
The term diabetes mellitus does not refer to a single disease, but rather encompasses a heterogeneous group of diseases that have in common a systemic disorder of carbohydrate metabolism. Although diabetic syndromes may be classified into a number of subclasses, each with a distinct pathogenesis and clinical symptoms, two major forms of the disease are generally recognized based on their dependency for insulin, a peptide hormone central to the regulation of carbohydrate metabolism synthesized by the $\beta$ cells of the pancreatic islets of Langerhans.

**Insulin-dependent diabetes mellitus (IDDM),** considered the classical form of diabetes, is often observed at a young age and has as its underlying pathogenesis an inability to synthesize insulin, thought to be due to the immunological destruction of islet $\beta$ cells. The precise nature of the factors involved in the aetiology of this immunological attack, however, still remain unclear. Onset of this syndrome is acute, and IDDM patients have an absolute requirement for exogenous insulin therapy for survival.

**Non-insulin dependent diabetes mellitus (NIDDM),** a milder form of diabetes, is generally observed at an older age and is thought to result from a relative insulin deficiency or a resistance to the biological activity of the hormone. NIDDM has a more chronic progression than IDDM and can often be controlled by diet and exercise or oral hypoglycaemic agents.

During the history of diabetes research, much attention has focused on identifying and describing animal models of the disease that simulate the human syndrome. Animal models allow opportunities to study many aspects of disease initiation and pathogenesis that are not possible in humans, and to date have provided much of our knowledge of the pathogenesis of a wide variety of human disorders, enabling the
development of strategies for disease development and control in several cases. While NIDDM has been found to occur naturally in a number of animal species, spontaneous IDDM is less common and there has been difficulty in finding a model that effectively simulates the human syndrome. For this reason a number of chemical, hormonal or infectious agents have been used to induce diabetes in animals not usually prone to the disease, and these have provided a valuable adjunct to the few available spontaneous models for the study of IDDM pathogenesis. To date however, no induced model has been available that avoids the possible adverse secondary effects of the chemical or infectious agents used for disease expression.

The following work describes the features of a newly-described diabetic syndrome induced in rats by manipulation of the immune system using a combination of thymectomy and irradiation (Tx-X), and examines the validity of using this model to simulate human IDDM. Detailed clinical, histopathological and immunological features of the syndrome are thus presented, and it is argued that this syndrome closely resembles human IDDM in many respects. Studies on the immunopathogenesis of Tx-X-induced IDDM suggest an autoimmune pathogenesis for the disease, while the consequences of Tx-X could be profoundly modulated by altering the hygienic background of the animals used. It is thus proposed that the Tx-X model is a relevant model of human IDDM and has potential for the study of diabetes initiation and pathogenesis.
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2.1 INTRODUCTION

2.1.1 Historical Perspective

Diabetes research has a history dating back to the late 17th century, when patients were noted to have polyuria ('diabetes' meaning literally to 'siphon-through') and their urine was observed to have a sweet taste. Thus, the classification *diabetes mellitus* ('honey' diabetes) was adopted and due to the associated polyuria the kidney was originally proposed as the causative organ. It was not until 1795, when the pancreas of a diabetic patient at autopsy was noticed to be atrophied and granular, that the first association of the pancreas with diabetes was made (Levine, 1979).

The course of diabetes research was changed in 1889 by Minkowski and von Mering, who performed the first effective total removal of the pancreas of a dog which led to raised blood glucose, glycosuria, polyuria, loss of weight and acetone in the urine, thus demonstrating a relationship between pancreatic function and *diabètes mellitus* (Minkowski, 1929). As no defects of the nervous system could be found in the pancreatectomized animal, the diabetic syndrome was attributed directly to removal of the pancreas. Although at the time these findings were considered contrary to popular belief (as several highly respected workers had apparently shown that pancreatectomy did not lead to a diabetic state), Minkowski and von Mering's results were repeated and ultimately accepted.

As a result of the above work and that of pathologists such as Paul Langerhans, who first described 'little heaps of cells' in rabbit pancreas, later termed the 'islets of Langerhans', it gradually became evident that the
pancreas must produce an internal secretion capable of controlling blood glucose levels, and that this secretion probably originated in the islets. From 1898 onwards attempts were made to isolate and purify this hypoglycaemic factor, but initial pancreatic extracts were so toxic they could not be used in live animals. In 1921, however, Banting and Best prepared a dog pancreatic extract that could lower the glycosuria of diabetic dogs. This extract, initially called *isletin*, later became known as *insulin* and was purified and successfully used to control hyperglycaemia in humans (Wrenshall, 1962). Insulin is now known to be a polypeptide hormone consisting of 51 amino acids in 2 polypeptide chains linked by disulphide bonds synthesized by the *B* cell of the pancreatic islets of Langerhans (Smith et al, 1983).

### 2.1.2 Types of Diabetic Syndromes

Today diabetes mellitus is classified into a number of subclasses depending on the severity of the disease and requirement for insulin therapy. The common theme for all subclasses, however, is the inability to control blood glucose levels as a result of a systemic disorder in carbohydrate metabolism. Type 1 or insulin-dependent diabetes mellitus (IDDM) is characterized by weight loss, proneness to ketosis and an absolute requirement for exogenous insulin therapy. This form of diabetes may be diagnosed at any age but is more common in the 17 years or younger age group (hence the previous "juvenile-onset" classification). Non-insulin-dependent diabetes mellitus (NIDDM), also known as mature-onset diabetes mellitus, is usually associated with obesity, but not insulin deficiency, and can often be controlled by oral hypoglycaemic agents or diet. This syndrome may also be diagnosed at any age, but tends to be more common in the 40 years and above age group (National Diabetes
Data Group, 1979). Other forms of diabetes mellitus include gestational diabetes associated with pregnancy and glucose intolerance associated with certain conditions or syndromes.

This review will focus on the current state of research relating to the aetiology and pathogenesis of IDDM, both in humans and in animal models of the human syndrome.

2.2 THE HUMAN IDDM SYNDROME

2.2.1 Clinical Features

IDDM is characterized clinically by an abrupt decline in circulating insulin levels and associated rapid onset of glucosuria and severe hyperglycaemia. At this stage patients are prone to ketosis as indicated by raised levels of circulating $\beta$-hydroxybutyrate and acetoacetate (ketotic-stage). Exogenous, parenteral insulin therapy initiated at this point is effective in controlling hyperglycaemia and ketosis and daily therapy is required for survival. A pre-ketotic stage may also be identified up to several months prior to the onset of ketosis based on a fasting euglycaemia but an inability to control blood glucose levels after oral administration of glucose (National Diabetes Data Group, 1979). Polydipsia, polyuria, polyphagia, hypercholesterolaemia and hypertriglyceridaemia are also characteristic features which may be present 1-2 months prior to the onset of the ketotic stage (Drash and Becker, 1978; Cahill and McDevitt, 1981). Long-term complications of IDDM include neuropathy, nephropathy, retinopathy, coronary heart
disease and peripheral vascular disease as a result of impaired neurological function and microcirculation (Taylor and Agius, 1988).

2.2.2 Incidence

Incidence levels of IDDM are difficult to ascertain precisely due to age, sex, seasonal and geographical variations and accuracy of disease diagnoses and record keeping. Thus, records of Western countries tend to be more readily available and accurately interpreted than those of less developed countries. Nevertheless, the available data suggests that incidence levels of IDDM world-wide, particularly in Northern Europe and North America, appear to be rising at a steady rate, with an estimated doubling rate of 20 to 30 years in some countries (Bingley and Gale, 1989a). A common feature of incidence rates of IDDM in most regions of the world is the geographical variation between, and even within, countries. This trend is particularly evident in Scandinavian countries, which have relatively stable populations and some of the highest incidence rates of IDDM in the world, ranging from 38 cases per 100 000 per year in Finland, to 25 cases per 100 000 per year in Sweden (Akerblom and Reunanen, 1985; Dahlquist et al., 1985). Similar figures have also been reported for Denmark, Norway and other European countries (Green and Anderson, 1983; Joner and Sovik, 1989; Bingley and Gale, 1989b). In Scotland, the figure drops to around 18 cases per 100 000 per year, to 10 in the Netherlands, England and Luxembourg and to 4 in France (Patterson et al., 1983; DeBeaufort et al., 1988; Bingley and Gale, 1989a).

In North America the figures range from 20 cases per 100 000 per year in Minnesota to 9 in California, while in Japan the figure is as low as 0.6
(Diabetes Epidemiology International Research Group, 1988). In Australia, a figure of 14 cases per 100,000 per year has been estimated, indicating a frequency of around 2,000 newly diagnosed cases of IDDM in this country every year (Karvonen et al., 1993).

A number of studies have also reported seasonal variations in the incidence levels of IDDM, with the number of reported cases reaching a peak during winter and declining dramatically during summer months (Fishbein et al., 1982; Dahlquist et al., 1985; Joner and Sovik, 1989). Incidence levels also tend to peak in certain age groups, particularly between the ages of 5 to 9 and 10 to 14 years (Fishbein et al., 1982; Joner and Sovik, 1989).

Generally there is little difference in incidence levels of IDDM between males and females world-wide, although a number of studies show a definite trend towards an increased incidence in males (Fishbein et al., 1982; Dahlquist et al., 1985; deBeaufort et al, 1988; Diabetes Epidemiology International Research Group, 1988; Joner and Sovik, 1989).

2.2.3 Histopathology

i) Pancreatic

The most significant pancreatic lesions of recent-onset IDDM patients are confined to the islets of Langerhans - clusters of cells dispersed throughout the pancreas which constitute the endocrine component of pancreatic function. Islets consist of 4 different cell types - $\alpha$, $\beta$, $\delta$ and PP cells. These cells synthesize glucagon, insulin, somatostatin and
polypeptide (PP) hormones respectively and are responsible for the correct regulation of carbohydrate metabolism.

Early morphological studies of pancreas sections taken from children who died at the onset of IDDM revealed a mononuclear-cell infiltration confined to the islets termed insulitis (Gepts, 1965). Later studies characterized this infiltration as consisting of activated (MHC class II and interleukin-2 receptor (IL-2r) positive) T lymphocytes, predominantly of the cytotoxic/suppressor (CD8+) and to a lesser extent helper/inducer (CD4+) and natural killer (NK) cell sub-populations (Bottazzo et al., 1985). Pancreatic isograft biopsies have tended to support these findings (Sibley et al., 1985), although one such study failed to detect any sign of insulitis in 7 pancreatic biopsies taken 2-4 months after the onset of disease (Hanafusa et al., 1990). When insulitis is present, however, most (but not usually all) islets are affected and this is accompanied by a B cell loss of up to 90 percent (Gepts, 1984; Gepts and LeCompte, 1985). This loss of B cell mass prior to the onset of diabetes indicates that there is an extended preclinical phase, and this is supported by clinical data showing a progressive loss of glucose-stimulated insulin secretion prior to disease onset (Eisenbarth, 1986).

In long-term IDDM patients a reduction in islet size (atrophy) results from the almost exclusive destruction of B cells. In severe cases (end-stage atrophy), such islets show no signs of cellular infiltration and consist entirely of normal or elevated numbers of α, δ and PP cells, with an almost complete absence of B cells (Gepts, 1977; Hanafusa et al., 1990). Consequently a severe reduction in insulin-producing capacity results, which manifests clinically as hypoinsulinaemia and associated
hyperglycaemia. Chronic IDDM can eventually lead to reduction in size and weight of the pancreas and fibrosis.

ii) Extrapancreatic

In addition to lesions of the pancreas, other pathological changes include glomeruloscleroses and other kidney changes, retinopathy, neuropathy, cardiovascular diseases such as atherosclerosis and microangiopathy, various endocrine defects and abnormal pituitary function (Legg and Harawi, 1985).

2.2.4 Immunopathology

The histopathological picture described above strongly suggests a role for the immune system in the destruction of $\beta$ cells and development of IDDM. In addition, the association of IDDM with other autoimmune diseases such as thyroid disorders (Grave's disease, Hashimoto's thyroiditis), Addison's disease, rheumatoid arthritis and myasthenia gravis provides circumstantial evidence for an autoimmune pathogenesis (Rossini et al., 1991). To support this hypothesis, there is evidence for both humoral and cell-mediated autoimmunity in IDDM patients.

i) Humoral Immunity

Islet-cell Cytoplasmic Antibodies (ICA)

Circulating autoantibodies to islet-cell cytoplasmic components were first described in 1974 as detected by indirect immunofluorescence of human serum on frozen sections of human type O pancreas (Bottazzo et al., 1974). These antibodies produced a uniform staining pattern across all
cell types of the islet, were shown to be of the immunoglobulin G (IgG) class and a proportion, but not all, were complement-fixing (Bottazzo et al., 1974; Bottazzo et al., 1980). Subsequently it has been shown that ICA can be detected in 60 to 90 percent of newly diagnosed IDDM patients and in 0.1 to 3 percent of the community (Timsit et al., 1992; Genovese et al., 1992; Irvine et al., 1977). The presence of ICA, however, seemed to be dependent upon the time elapsed since diagnosis, being detected in 20 percent of patients 2 to 5 years after diagnosis and dropping to 5 percent after 10 to 20 years, presumably due to a loss of sustaining antigen (Irvine et al., 1977).

Since the detection of ICA, attention has focused on their ability to predict the onset of IDDM and today they have become the best serological marker of disease onset. Studies have shown that high titres of ICA (>40 Juvenile Diabetes Foundation [JDF] units) of the complement-fixing, IgG class have a high predictive value within families of IDDM patients and that lower levels (<20 JDF units) are predictive in non-familial IDDM (Bonifacio et al., 1990a; Landin-Olsen et al., 1989). In addition, loss of $\beta$ cell function in IDDM patients, as assessed by $\beta$ cell C-peptide release, has been shown to be more rapid and complete in ICA positive than in ICA negative patients (Peig et al., 1989).

Despite the high predictive value of ICA, a high proportion of ICA positive patients do not progress to overt diabetes (Landin-Olsen et al., 1989; Bosi et al., 1991). The predictive value of ICA may be enhanced when considered in conjunction with insulin autoantibodies (IAA), however this remains controversial (Dean et al., 1986; Betterle et al., 1987; Wilkin, 1990). Recent improvements in the immunofluorescent techniques have enabled the detection of a heterogeneous population of ICA in IDDM
patients. Thus, in addition to the classical "whole islet" staining, ICA from a proportion of recent-onset IDDM patients showed a "granular" pattern where staining was $\beta$ cell-specific, or a "mixed" pattern in which both $\alpha$ and $\beta$ cells were stained, although the $\beta$ cells more intensely (Genovese et al., 1992; Timsit et al., 1992). Ninety percent of sera from newly diagnosed IDDM patients and 70 percent of sera from endocrine autoimmune patients who developed IDDM showed the "whole islet" pattern, while both the granular or mixed patterns were rarely seen (Genovese et al., 1992). Therefore, this heterogeneity in ICA staining may allow a greater degree of prediction for development of IDDM.

64 Kilodalton (64kDa) Autoantibodies

Despite the widespread use of immunofluorescence to detect ICA in IDDM patients, problems are associated with standardization, sensitivity and reproducibility of the technique (Bonifacio et al., 1990b) and this has lead workers to adopt alternative approaches to detect autoantibodies to islet cytoplasmic components. One such approach has been to metabolically label islet cell proteins in vitro with a radioactively-labelled amino acid, such as $^{35}$S-methionine, and then search for immunoprecipitating antibodies to the resulting radiolabelled antigens of islet cell extracts. By using this technique, Baekkeskov and associates were able to detect autoantibodies in recent-onset IDDM patients to an islet cell component of Mr 64 000 daltons (64kDa), as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography of isolated antibody/antigen complexes (Baekkeskov et al., 1982).

A subsequent study identified 64kDa autoantibodies in 80 percent of newly diagnosed IDDM patients and only 3 percent of healthy controls (Sigurdsson and Baekkeskov, 1990). In another study using canine islets,
Barmeier et al., identified 64kDa antibodies in 89 percent of recent-onset patients (Barmeier et al., 1992). Similar to ICA, levels of 64kDa antibodies tend to decline with time from disease onset, reaching low or undetectable levels after 18 months (Sigurdsson and Baekkeskov, 1990). To assess the value of 64kDa antibodies in predicting future onset of IDDM, these workers also examined sera from pre-diabetic individuals at "high-risk" for IDDM (monozygotic twins or siblings to IDDM patients) who subsequently went on to develop disease (Baekkeskov et al., 1987). Sixty four kDa antibodies were detected in 78 percent of serum samples taken from IDDM patients 4 to 91 months prior to disease onset, and in 100 percent of first degree relatives. In addition, 64 percent of 64kDa positive patients were concordant for ICA.

Other studies have shown that the combined measurement of 64kDa antibodies and ICA have an increased ability to predict IDDM onset when compared to ICA alone (Seibler et al., 1992). Christie and associates have shown that the predictive value of 64kDa antibodies may be improved even further if tryptic fragments of the antigen are used (Christie et al., 1990a). Thus, 93 percent of IDDM patients immunoprecipitated one of three fragments of the 64kDa antigen obtained by tryptic digestion, and some previously 64kDa antibody negative patients had antibodies to one of these fragments.

The immunoprecipitation assay, although providing high sensitivity and specificity, is costly, time consuming and not suitable for screening large numbers of samples. An alternative approach is to subject islet-cell extracts to electrophoretic separation and transfer the separated proteins to a solid membrane matrix. Serum samples are then immunoblotted against the membrane-bound antigens and bound antibodies detected by
means of a secondary, enzyme-conjugated antiserum in conjunction with a coloured or luminescent enzyme substrate. Unfortunately, there are few reports of success with this technique in relation to IDDM, but in the two reports of immunoblotting of human IDDM sera against islet cell preparations, 71 percent of sera from recent-onset IDDM patients showed high titre antibodies to human islet cell antigens, while 29 percent of cases were positive when the RINm5 rat insulinoma cell line was used as antigen (Karounos et al., 1990; Karounos and Thomas, 1990). Although a dominant protein band of 52kDa was identified in RINm5 extracts, the pattern of reactivity was heterogeneous when human islets were used, with proteins in the range of 52 to 150kDa being detected and no single band being bound in more than 50 percent of cases. These results are contradicted by those of Tuck and associates, however, who failed to detect autoantibody reactivity to RINm5 extracts by this method (Tuck et al., 1992).

Islet Cell Surface Antibodies (ICSA)
In addition to ICA, antibodies directed towards cell surface components of viable islets have also been demonstrated in IDDM sera. Indirect immunofluorescence, complement-mediated cytotoxicity assays and radioimmunoassays have been used to detect antibodies to viable rat or mouse islet cells in 35 to 45 percent of IDDM patients (Lernmark et al., 1978; Lernmark et al., 1980; Huen et al., 1983; Toguchi et al., 1985). As seen with ICA, levels of ICSA seem to decline with duration of disease and in addition, appear to be preferentially lytic to B cells, indicating an active role for this type of antibody in B-cell destruction (Lernmark et al., 1980; Toguchi et al., 1985). The value of this type of antibody in predicting the onset of IDDM, however, remains to be determined.
Insulin Autoantibodies (IAA)

For many years autoantibodies to insulin have been known to develop following the initiation of insulin therapy (Berson et al., 1956). More recently, however, IAA have been detected in the serum of patients at the onset of IDDM but before the commencement of insulin therapy (Palmer et al., 1983) and consequently, interest in the value of IAA as predictive markers of IDDM has been renewed.

IAA have been detected by radioimmunoassay in 30 to 50 percent of newly diagnosed IDDM patients and in 30 to 50 percent of subjects at "high-risk" for the development of IDDM - that is, ICA positive monozygotic twins and first degree relatives of IDDM patients (Srikanta et al., 1986; Ludwig et al., 1987; Vardi et al., 1987). In one study, the detection of IAA in conjunction with ICA appeared to have a greater predictive value for progression to IDDM than for either marker alone (Srikanta et al., 1986).

In contrast, other studies have not shown this association, but rather have shown that IAA are prevalent in patients with on-going organ or non-organ specific autoimmune diseases such as autoimmune thyroid disease, Addison's disease, pernicious anaemia and systemic lupus erythematos (Wilkin et al., 1985a; Ludvigsson et al., 1988; DiMario et al., 1990). An inherent problem with the use of IAA as predictors of IDDM appears to be the specificity and sensitivity of the radioimmunoassay (Wilkin et al., 1985b; Sodoyez-Goffaux et al., 1988). This fact, in conjunction with the apparent lack of disease specificity of IAA restricts the use of these antibodies, whether alone or in conjunction with ICA, as predictive markers of IDDM.
Other Antibody Specificities

In addition to antibodies to islet cell components and insulin, serum samples from IDDM patients have been shown to contain antibody reactivity to a number of other self and non-self antigens. A high proportion contain antibodies to thyroid components, parietal cells, adrenal gland cells, anterior pituitary cells, tubulin, single-stranded DNA and insulin receptor (Nerup and Lernmark, 1981; Drell and Notkins, 1987; Ludwig et al., 1987; Johnston et al., 1990). Antibody reactivity has also been detected to immunoglobulin, lymphocyte cell surface antigens, human albumin, bovine albumin and viruses such as Coxsackievirus B and Reovirus (Drell and Notkins, 1987; DiMario et al., 1988; Mangili et al., 1988; Segers et al., 1989; Karjalainen et al., 1992a).

ii) Cell-mediated Immunity

The first evidence for abnormalities in cell-mediated immunity in IDDM patients was provided in 1971 by Nerup and associates using leukocyte migration studies (Nerup et al., 1971). This study demonstrated that in vitro migration of peripheral blood mononuclear cells (PBMNC) could be significantly inhibited by a porcine pancreatic extract in 55 percent of patients (regardless of disease duration) when compared to normal controls. The inhibition was organ specific in that liver or kidney extract caused no inhibition, and was not a result of insulin in the preparation as neither porcine or bovine insulin caused inhibition. The authors concluded that an anti-pancreatic cellular hypersensitivity could be demonstrated in IDDM patients, but due to the nature of the study antigenic activity could only be ascribed to the pancreas as a whole and not to islet or β cell components.
Evidence for an anti-β cell, cell-mediated immune response was subsequently provided by in vitro studies showing strong adherence (but not cytotoxicity) of PBMNCs from IDDM patients to human or rat insulinoma cell lines (Huang and MacLaren, 1976; Lang et al., 1987). The adherent cell population was shown to consist predominantly of Ia+ (activated), CD4+ T lymphocytes (Lang et al., 1987; Segain et al., 1988). More significantly, other studies have demonstrated that PBMNC are actually cytotoxic to β cells in vitro. Using either human, mouse or rat β cells as targets, it has been shown that PBMNC isolated from IDDM patients can inhibit β cell function in vitro, as assessed by insulin release of co-cultured islets, and immunochemical analysis has shown that CD8+ lymphocytes are responsible (Lohmann et al., 1986; Lampeter et al., 1987). Cytolytic activity has also been shown, as assessed by radioisotope release from lyzed 51-Cr-labelled β cells, either directly or via antibody mediated mechanisms (Charles et al., 1983). In addition, PBMNC from healthy subjects are able to inhibit the β cell cytotoxic effects of PBMNC from IDDM patients, and this suppressive effect is mediated by CD8+ T lymphocytes (Lohmann et al., 1986; Krug et al., 1991).

Additional evidence for lymphocyte hypersensitivity towards islet cells in IDDM is provided by recent lymphocyte proliferation studies. By measuring DNA synthesis as an indicator of lymphocyte activation and proliferation, 68 percent of preclinical IDDM and 41 percent of recent-onset IDDM patients showed significant PBMNC proliferative responses to sonicated foetal porcine pro-islets when compared to normal control subjects (Harrison et al., 1992a). An additional study has shown that PBMNC from 100 percent of preclinical and 64 percent of clinical IDDM patients respond to intact human or porcine islets, as measured by DNA synthesis incorporation and cytokine (IFN γ, IL-4, GM-CSF) production
(Harrison et al., 1991a; Morse et al., 1992). The response was tissue specific, in that there was no response to thyroid, liver or kidney antigens and was shown to be greater than the response to insulin alone.

In addition to cytotoxicity and proliferation assay studies, studies of peripheral blood T lymphocyte subsets from IDDM patients tend to support the concept of an irregularity in T cell function or immunoregulation. An increased number of activated peripheral T cells seems to be a consistent feature of IDDM (Jackson et al., 1982; Pozzilli et al., 1983; Hayward and Herberger, 1984) and abnormalities of CD4+ and CD8+ T cell subsets have been reported, but tend to be somewhat conflicting. Thus, Pozzilli and associates reported levels of total peripheral T lymphocytes in newly-diagnosed IDDM patients equivalent to those of normal controls, but the proportion of these cells declined significantly as the disease progressed. The proportion of CD4+ helper/inducer T cells followed the same trend as total T cells, but a relative increase in cytotoxic and decrease in "suppressive" CD8+ T cells was observed in both newly diagnosed and long-standing IDDM patients (Pozzilli et al., 1983).

These observations are supported by the work of Herold and co-workers who also showed alterations of the CD4+/CD8+ cell ratio in the initial stages of disease (Herold et al., 1984), but are in contrast to the work of Hayward and Herberger, who demonstrated a marked increase in the total number of peripheral T lymphocytes at IDDM onset which decreased to near-normal levels as disease progressed (Hayward and Herberger, 1984). This study also reported no alterations in the CD4+/CD8+ ratio, both in newly diagnosed and long-term patients. Faustman and associates also noted a normal CD4+/CD8+ ratio in newly diagnosed patients, but this ratio was depressed in pre-diabetic and long-term
diabetic patients (Faustman et al., 1989). These workers also noted elevated levels of CD4+ T cells expressing the CD45R antigen, a marker of suppressor-inducer cells, when compared to cells expressing the CDw29 antigen (helper-inducer cells) in pre-diabetic patients, but this ratio tended to approach normal levels in recent-onset and long-term diabetic patients.

Together these studies suggest that the relative proportions of lymphocyte sub-populations in diabetic patients are in a dynamic state during the course of the disease, and it is possible that identification of a particular lymphocyte abnormality or imbalance at an early stage of the disease may allow prediction of disease onset.

In addition to alterations in the T lymphocyte subset, irregularities in the non-T cell subset of PBMNC from IDDM patients have been reported. In particular, natural killer (NK) cell numbers are decreased in newly diagnosed IDDM patients as compared to NIDDM and normal controls (Negishi et al., 1986; Hussain et al., 1987). NK cells of IDDM patients also show a decrease in cytolytic activity as assessed by the 51Cr release assay using human myeloid (K562) cells as targets, but increased cytolytic activity when islet cells are used as targets (Negishi et al., 1986). This effect is only seen at the onset of IDDM, however, and is not demonstrable in long-term patients.

Increased numbers of activated cells of the monocyte-macrophage sub-populations of PBMNC have also been demonstrated in recent-onset IDDM patients. Thus, the number of cells positive for the Leu M3 (monocyte/macrophage) marker, in conjunction with the CD25 (IL-2 receptor) or DR (MHC class II) markers, were significantly higher in newly-
diagnosed IDDM patients when compared to normal controls and again, this effect was not seen in long-term IDDM patients (Nicoletti et al., 1991).

2.2.5 Genetics

It has long been recognized that IDDM occurs in families more frequently than in the general population, and numerous family studies have attempted to confirm this. Despite the possible inconsistencies in the diagnosis of different categories of diabetes when tracing the history of the disease within a family, these studies indicate a recurrence risk among first degree relatives of IDDM probands of between 3 and 6 percent, as compared to 0.4 percent for the general population (Kobberling and Bruggeboes, 1980; Tillil and Kobberling, 1987; Green, 1990). In addition, this risk is significantly greater if the father is the affected parent (Dahlquist et al., 1985; Tillil and Kobberling, 1987). An increased risk is also seen in second degree relatives (1.5 percent), but this is somewhat less than first degree relatives (Green, 1990).

Of particular interest is a study examining concordance rates for IDDM in identical (monozygotic) twins (Barnett et al., 1981). Although biased in the favour of concordant pairs, this study showed a concordance rate of around 55 percent, indicating that although susceptibility to IDDM is determined genetically, non-genetically determined factors are as equally important in the aetiology of the disease.

More recently, effort has focused on identifying genetic markers that indicate susceptibility to disease, and these will be discussed later in this review.
2.3 ANIMAL MODELS OF DIABETES

For the study of IDDM or any human disease at the pathological or therapeutic level, human experimentation is often not feasible for both ethical and practical reasons. Experimental animal models that closely resemble the human disease are therefore essential in order to characterize and understand the complex aetiological mechanisms that are often involved. An excellent example of the value of animal models in the study of human disease is provided by the discovery of pancreatic diabetes (see section 2.1.1) where studies on the dog were essential for establishing the role of the pancreas in disease induction and also for isolating and testing the first preparations of insulin that would later lead to its therapeutic use in man. The search for alternative animal models of IDDM has identified a number of spontaneously occurring and experimentally induced syndromes, predominantly in rodents, that have provided an extremely valuable source of experimental data.

2.3.1 Spontaneous Models

It has long been recognized that spontaneous diabetes in animals is a common event, having been first observed in monkeys in the mid-19th century, although the majority of these syndromes resemble the non-insulin form of human diabetes (NIDDM) in that they display obesity, hypoinsulinaemia and resistance to insulin. Nevertheless, syndromes equivalent to human IDDM (that is, non-obese, hypoinsulinaemic, ketoacidotic syndromes) do occur spontaneously in rodents (reviewed in Mordes and Rossini, 1981) and of these, the Bio-Breeding (BB) rat and non-obese diabetic (NOD) mouse are now the most commonly used and thus have been extensively characterized.
i) The BB Rat

The IDDM syndrome of the BB rat was first discovered as a spontaneous mutation of the Wistar-derived rat at the Bio-Breeding Laboratories of Canada and at the University of Massachusetts, Worcester, Massachusetts (Nakhooda et al., 1977). This original colony was designated BB/W as referring to the Worcester colony, but colonies established in different centres are now given designations relating to that centre, for example, BB/E for the Edinburgh colony (Marliss, 1983). In addition, a second line of BB rats resistant to diabetes (DR-BB) was also established in parallel to the original diabetes-prone (DP-BB) line, and this line now provides a valuable source of control animals (Joseph et al., 1993). The acute syndrome described in the DP-BB rat is metabolically and pathophysiologically similar to IDDM of man and has been extensively reviewed (Nakhooda et al., 1978; Seemayer et al., 1980; Crisa et al., 1992).

Clinical Features

The diabetic syndrome of the DP-BB rat is characterized by hyperglycaemia ranging from 14 to 40 mM, hypoinsulinaemia in the range of 0 to 1 ng/ml and hyperketonaemia of up to 13 mM (Nakhooda et al., 1978; Seemayer et al., 1982). A spectrum of disease severity is observed depending on the degree of ketoacidosis. Severely ketoacidotic rats (blood ketone levels between 6 and 13mM) show dehydration and rapid weight loss, while moderately ketoacidotic rats (1 to 5mM) lose weight gradually. A third (stable) group, with ketonaemia of less than 1mM, maintains weight for over 40 days (Nakhooda et al., 1977). Severely ketotic rats die within two weeks unless treated with insulin and all rats show polyuria and glycosuria of varying degrees.
**Time of Onset and Incidence of Disease**

Onset of disease in DP-BB rats is usually between 48 and 120 days of age (mean 67 days) and is extremely rapid. Eighty to 90 percent of animals become affected and similar incidence rates are seen for males and females (Nakhooda et al., 1978). The cumulative frequency of diabetes in the DR subline is less than 1 percent (Crisa et al., 1992).

**Histopathology**

Histological examination of the pancreas shows an intense insulitis with an associated reduction in islet size (atrophy) and $\beta$ cell number (Nakhooda et al., 1977; Seemayer et al., 1982). Simultaneous insulitis and islet atrophy are unique to the BB rat model and show similarity to the human situation. Islets of recent-onset diabetic rats show extensive infiltration by activated lymphocytes and macrophages, with $\beta$ cell necrosis and degranulation. After a few days of overt diabetes, insulitis cannot be observed, islets decrease in number and size and $\beta$ cells are absent ("end stage" islet atrophy) (Seemayer et al., 1982; Marliss et al., 1983). Evidence also suggests that in overtly diabetic rats there is a reduction in $\alpha$ and $\delta$ cell numbers and a decrease in glucagon and somatostatin concentrations (Seemayer et al., 1982).

Examination of the onset and progression of disease by pancreatic biopsy revealed that in the pre-diabetic phase, islet lesions develop rapidly about 2 to 3 weeks before onset of overt disease (Logothetopoulos et al., 1984). Lesions start focally in a small number of islets and most are infiltrated within a few days. Immunofluorescent analysis of sequential pancreas sections taken from DP-BB rats between 30 and 110 days of age identified macrophages (ED1+, MHC Class II+) as being the first cells to infiltrate the islet, approximately 3 weeks before IDDM onset (Walker et
Significant numbers of activated CD4+ T-lymphocytes and NK cells are the next to infiltrate, followed by a massive number of B-lymphocytes approximately 10 to 14 days before the onset of disease (Dean et al., 1985; Walker et al., 1988; Hanenberg et al., 1989).

Lymphocytic thyroiditis is also a common feature of the BB rat strain in general, and of diabetic rats in particular. One study has demonstrated lymphocytic thyroiditis in 60 percent of diabetic BB/W rats and 11 percent of non-diabetic cohorts (Sternthal et al., 1981), and others have reported incidence levels of 30 to 60 percent by 90 days of age for the BB/W colony as a whole (McKeever et al., 1990). Within sublines of the BB/W colony, there appears to be a variation in the incidence of thyroiditis, ranging from 100 percent in NB to only 5 percent in BE sublines (Rajatanavin et al., 1991).

In addition to endocrine lesions, BB rats are more susceptible to pulmonary infections, sterile lymph node granulomata, kidney and pancreas granulomas, lymphomas and prostatic atrophy (Wright et al., 1983). B-cell lymphomas of mesenteric lymph nodes are particularly prevalent in long term diabetic rats (Crisa et al., 1992).

Humoral Immunity
Evidence for a role for humoral autoimmunity in the pathogenesis of IDDM in BB rats is provided by the ability to detect antibodies to islet cell surface components (ICSA) in these rats before and after disease onset. Increased levels of ICSA can be detected in approximately 25 percent of animals at 60 days of age, and the incidence levels steadily increase until
reaching a peak of around 75 to 85 percent at 100 days of age (Dyrberg et al., 1982; Dean et al., 1987). A higher incidence of ICSA is also observed in rats becoming diabetic at a younger age (less than 85 days) than those developing the disease at a later age (Pipeleers et al., 1987). These antibodies have been shown to fix complement and mediate cytolytic activity against islet cells *in vitro* (Martin and Logothetopoulos, 1984). Islet cytolytic activity can be detected in the sera of prediabetic rats from 40 days and peaks at around 95 days of age - a few days before disease onset in most cases (Laborie et al., 1985). A pathogenic role for ICSA in BB rats has not been described *in vivo*, however, and thus they may possibly be generated in response to prior islet-cell damage.

Of particular interest is that a number of studies have failed to detect ICA in BB rats (Like et al., 1982a; Elder et al., 1982; Maclaren et al., 1983; Dyrberg et al., 1983). In contrast, antibodies to the human 64kDa islet cell antigen (see section 2.2.4 (i)) have been detected in a high proportion of sera from diabetic and prediabetic BB rats by immunoprecipitation (Baekkeskov et al., 1984). These antibodies are detectable from a very early age and seem to have a high predictive value for disease onset. In addition, Cole and associates have reported a low frequency of antibodies to a 64kDa islet protein as detected by immunoblotting (Cole et al., 1992).

Circulating antibodies to insulin (IAA) have been demonstrated, but these findings are controversial. Low levels of IAA's have been reported as occurring in 30 to 70 percent of diabetic BB rats by enzyme-linked immunosorbent assay (ELISA), depending on the type of insulin used as antigen (rat or human), but seem to be poor predictors of disease onset and do not correlate with insulitis (Dean et al., 1987; Diaz et al., 1989; Diaz et al., 1991). This is in contrast to the work of Markholst and
associates, who failed to detect significant levels of IAA's in the sera of prediabetic rats using both ELISA and the more sensitive RIA techniques (Markholst et al., 1990).

Similar to human IDDM, circulating antibodies reactive to lymphocytes, thymocytes, thyroid colloid antigens, smooth muscle and gastric parietal cells have been detected in BB rats, providing evidence for a generalized dysfunction of immunoregulation in these animals (Like et al., 1982a; Dyrberg et al., 1982; Dyrberg et al., 1983; Maclaren et al., 1983).

**Cellular Immunity**

The primary perturbance of cell-mediated immunity in the DP-BB rat is a profound lymphopaenia. In the periphery, the lymphopaenia is confined primarily to T cells (W3/13\(^+\)), and is most striking in the T helper (W3/25\(^+\)) subpopulation, which shows a tenfold reduction in cell numbers compared to the control rat strain (Jackson et al., 1981; Jackson et al., 1983). Although OX8\(^+\) cells are present, the cytotoxic/suppressor (OX8\(^+\)/OX19\(^+\)) subpopulation is almost completely absent, while there is an increase in NK (OX8\(^+\)/OX19\(^-\)) cell numbers (Woda et al., 1986) and there is an inversion of the W3/25 to OX8 ratio to a value of 0.7 compared to 1.2 in controls (Elder and Maclaren, 1983). B cell numbers appear normal, but the number of activated (Ia\(^+\)) cell numbers are increased (Jackson et al., 1981). In the spleen, increased numbers of NK cells, macrophages and activated T cells are observed and also increased percentages of CD4\(^-\)CD8\(^-\)TCR\(^+\) (double negative) T cells, suggesting abnormal thymic maturation in BB-DP rats (Hosszufalusi et al., 1992). Studies of the onset of lymphopaenia in DP-BB rats show that total lymphocyte and T cell numbers are reduced at birth, both in the W3/25 and OX8 subsets, and there is an abnormal rise in cell numbers with time (Yale et al., 1985).
Greiner and associates have shown that the lymphopaenia of DP-BB rats is due almost entirely to the absence of T cells expressing the RT6 differentiation alloantigen (Greiner et al., 1986). The RT6 alloantigenic system consists of nonglycosylated or variably glycosylated 24-26 kDa peptides expressed only on the surface of mature peripheral T cells and not on thymocytes or bone marrow cells, and expression is restricted to the final stages of post-thymic T cell development (Koch et al., 1990; Crisa et al., 1990). The RT6 antigenic system consists of two allelic forms, termed RT6.1 and RT6.2, covalently linked to cell membranes by a phosphatidylinositol moiety (Koch et al., 1988). RT6+ lymphocytes constitute around 60 percent of peripheral T cells, identifying approximately 50 percent of W3/25+ and 70 percent of OX8+ cells, and have been shown to participate in graft versus host responses, mitogenic responses and in allogeneic stimulation in mixed lymphocyte reactions (Greiner et al., 1987).

Evidence for a regulatory role for RT6+ T cells in determining susceptibility or resistance to diabetes in BB rats was initially provided by the fact that diabetes could be prevented in DP rats by the transfusion of DR lymphoid cells, which contain normal levels of RT6+ cells (Rossini et al., 1984). More convincing evidence was provided by Greiner and associates in 1987, who were able to show that in vivo depletion of RT6.1+ T cells in young DR-BB rats induced overt IDDM in 50 percent of animals, and insulitis in 40 percent of treated animals that did not become diabetic (Greiner et al., 1987). Spleen cells isolated from non-diabetic, RT6.1 depleted DR-BB rats were also shown to transfer disease to DR and young DP rats. RT6 depletion of DR-BB rats has subsequently proved to be useful animal model of IDDM, providing an adjunct to the spontaneous syndrome of DP rats.
In addition to lymphopaenia, DP-BB rats have been shown to have poor splenic lymphocyte proliferative response to both mitogens and alloantigens. Removal of adherent cells from cultures or purification of T cells, however, restores this proliferative capacity, suggesting the presence of an inhibitory cell population (probably a macrophage) capable of suppressing lymphocyte activity \textit{in vitro} (Prud'homme et al., 1984; Woda and Padden, 1986). Additional functional studies have demonstrated defective cytotoxic activity to alloantigens (Prud'homme et al., 1988; Woda and Padden, 1987), however \textit{in vitro} cytotoxic activity of splenic lymphoid cells towards islet $\beta$ cells has been described (MacKay et al., 1985).

\textbf{ii) The NOD Mouse}

Another extensively characterized spontaneous IDDM syndrome occurring in animals is that of the non-obese diabetic (NOD) mouse. The progenitor mouse of this strain was discovered in a non-cataract prone, euglycaemic subline of a line of mice (CTL) that develop cataracts, and selective inbreeding of diabetic mice that showed good reproductivity produced the NOD strain (Makino et al., 1980). At the same time, however, a slightly hyperglycaemic subline of CTL mice was being established, from which a "control" strain for NOD mice termed nonobese nondiabetic (NON) mice was developed.

\textbf{Clinical Features}

Diabetic NOD mice display the classic features of human IDDM in that they develop hyperglycaemia (>16mM), hypoinsulinaemia (<1.0$\mu$U/ml), glycosuria, hypercholesterolaemia, ketonuria, polydipsia, polyuria and polyphagia (Makino et al., 1980). The disease process in NOD mice,
however, appears to be more chronic than that of humans or BB rats. In this regard, although the onset of disease appears abrupt, prospective analysis of changes in blood glucose levels shows a gradual rise, starting from 3 to 4 weeks before disease onset (Leiter et al., 1987). In contrast to humans and BB rats, NOD mice can survive without insulin therapy at onset of hyperglycaemia and for 1 to 2 months after disease onset before becoming insulin dependent (Leiter et al., 1987; Kikutani and Makino, 1992).

**Time of Onset and Incidence of Disease**
Marked sex differences are observed in both the pattern of disease onset and incidence levels of the disease. In females, onset of diabetes is observed at around 10 weeks of age and the number of affected animals increases steadily until reaching a peak at 30 weeks of age. In males, disease onset does not occur until around 20 weeks of age and far fewer animals are affected by 30 weeks of age when compared to females (Makino et al., 1980). As for BB rats, incidence levels of disease vary between the various colonies of animals. Thus, in the original NOD/Shi colony maintained in Japan, 70 to 80 percent of females and 20 percent of males develop disease (Kikutani and Makino, 1992). In the NOD/Wehi colony maintained in Australia, incidence of diabetes is 40 to 50 percent in females and less than 10 percent in males, while in the NOD/Lt strain originating from the USA but maintained in the same environment, 90 percent of females and 20 percent of males develop the disease (Baxter et al., 1991).

**Histopathology**
Histopathological examination of the pancreas of NOD mice reveals extensive insulitis or islet atrophy depending on disease duration. Insulitis
is first observed at around 3 weeks of age with high frequency in both male and female mice by 6 weeks of age and reaches a cumulative incidence of 80 to 90 percent by 13 weeks of age (Miyazaki et al., 1985). Time course analysis of cells infiltrating the islet indicate that from the earliest stages the predominant cell type is of the Thy1.2+ (T lymphocytes) / L3T4+(helper/inducer) phenotype, with lower numbers of Lyt 2+ (cytotoxic) subpopulations. The majority of cells are MHC class II positive, and a lower percentage express IL-2 receptor (Signore et al., 1989). These findings are in concordance with those of Miyazaki et al., who also found lower numbers of NK cells and B lymphocytes in the lesion (Miyazaki et al., 1985) and Formby and associates (Formby et al., 1992a) but are in contrast to those of Kanazawa and associates, who identified IgM+ B lymphocytes as the predominant infiltrating cell type (Kanazawa et al., 1984). Pancreatic sections from chronically diabetic mice generally show no sign of insulitis and islets consist mainly of functional non-β cell types (Ohneda et al., 1984; Leiter et al., 1987) consistent with the conclusion that the cytotoxic activity of infiltrating lymphoid cells is β cell specific.

Extrapancreatic lesions of NOD mice include mononuclear cell infiltration of the submandibular and lacrimal glands in mice older than 9 weeks (Kikutani and Makino, 1992) and lymphocytic thyroiditis in diabetic animals (Bernard et al., 1992a).

**Humoral Immunity**

NOD mice have been shown to produce circulating antibodies to both islet cell surface and cytoplasmic components. Approximately 50 percent of animals develop ICA by 2 weeks of age, and this proportion only increases slightly to a peak of 60 percent by 4 weeks of age (Reddy et al.,
1988). The majority of these antibodies show a "whole islet" staining pattern by immunofluorescence similar to that seen with human sera (Reddy et al., 1988; Reddy et al., 1990). ICSA reactivity is also common in NOD mice, being detectable by 6 weeks of age and reaching a maximum incidence of 50 percent by 12 to 18 weeks of age (Kanazawa et al., 1984). These antibodies appear to be able to fix complement and are cytotoxic to islet cells in vitro (Hari et al., 1986).

An interesting feature of anti-islet cell antibodies produced by NOD mice is their ability to be readily detected by immunoblotting using islet cells as antigen, a phenomenon not easily observed humans and not at all in BB rats (see sections 2.2.4(i) and 2.3.1(i)). Karounos and Thomas were able to identify antibodies to a 52 kDa component of RINm5F cells using this method, and these antibodies appeared to be present in all sera tested, whether from diabetic or non-diabetic NOD animals (Karounos and Thomas, 1990). These antibodies were tissue specific in that they did not react with kidney, liver or adipose cells, were not observed in NON control animals and appeared to have a high predictive value for disease onset. A similar study using both RINm5F and mouse islets as antigen identified reactivity to a 58 kDa islet cell component in all NOD sera tested, but not in control mice and not to non-islet tissues (Villa et al., 1992). These antibodies were present from 6 weeks of age and seemed to correlate with the onset of insulitis.

Immunoprecipitation analysis also revealed antibody reactivity in NOD sera to a 64 kDa islet cell component similar to that detected by human and BB rat sera (Atkinson and Maclaren, 1988). Such antibodies were present in 60 to 80 percent of NOD mice from an early age, but their prevalence declined rapidly after onset of disease.
IAA also appear to be present in NOD mice, but reported incidence levels are conflicting. In one study, IAA were readily detected in a large proportion of young mice (2 to 5 weeks old) and their frequency increased to 100 percent in older mice (Reddy et al., 1988; Michel et al., 1989). Maruyama and associates also reported a high frequency of IAAs in 12 to 14 week old mice correlating with the onset of insulitis (Maruyama et al., 1989). In contrast, Bernard and associates reported only 5 percent positivity in 4 week old animals and maximum incidence levels of 14 to 20 percent in animals 8 weeks or older (Bernard et al., 1992b). As for human IDDM, detection of IAA in NOD mice appears to poorly predict the onset of clinical diabetes but their predictive value may be increased when considered in conjunction with ICA (Reddy et al., 1990), however Ziegler and associates, using a sensitive fluid-phase radioimmunoassay, showed the presence of IAA alone to have a predictive value of up to 70 percent (Ziegler et al., 1989).

In addition to reactivity towards islet cell antigens and insulin, antibody reactivity has also been detected towards thyroid membrane components (Bernard et al., 1992a; Bernard et al., 1992b) and lymphocyte surface components (Kanazawa et al., 1984) in NOD mice.

**Cellular Immunity**

Although adult NOD mice are not lymphopaenic, abnormalities in peripheral, splenic and thymic lymphocyte populations have been reported. Reduced numbers of peripheral T cells have been demonstrated in young NOD mice (less than 12 weeks old), but these increase to normal or raised levels in older mice (Kataoka et al., 1983). Increased levels of the helper/inducer (L3T4\(^+\)) and cytotoxic (Lyt 2\(^+\)) phenotypes (but no alteration in the L3T4/Lyt2 ratio) have also been
demonstrated in mice of all ages (Kataoka et al., 1983; Pontesilli et al., 1987). In contrast, analysis of peripheral lymph node cell populations at disease onset revealed a significant reduction in the number of CD4+ cells and cells expressing the CD4+Vβ 8.1+ phenotype (Zipris et al., 1991).

In the spleen, diabetic NOD mice have been shown to have dramatically increased numbers of CD4+8-Thy1.2+ cells and CD4+Vβ 8.1, 8.2 and 8.3 positive T cells when compared to non-diabetic and non-NOD controls (Formby et al., 1992a). In the thymus, decreased CD4+CD8+ (double positive) and correspondingly increased CD4+CD8- (double negative) T cell numbers are observed prior to and after disease onset (Zipris et al., 1991).

Other cellular abnormalities include decreased NK cell activity, increased B lymphocyte activity and enhanced in vitro cell-mediated cytotoxicity to isolated islet cells (Kataoka et al., 1983; Maruyama et al., 1984), although this has been found to be a general feature of non-diabetes prone mouse strains (Burtles et al., 1992).

A comparison of the major features of human, BB rat and NOD mouse diabetic syndromes is shown in Table 2.1.

iii) Other Spontaneous Models

Other spontaneous-onset, non-obese diabetic syndromes of animals have been described that present interesting variations from the above models. These include such diverse species as the Chinese Hamster (Gerritsen and Dulin, 1967; Gerritsen et al., 1974; Like et al., 1974; Schmidt et al., 1970), Celebes Ape (Howard, 1978; Howard, 1986; Howard and van
Table 2.1. Comparison of the human, BB rat and NOD mouse diabetic syndromes.

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<thead>
<tr>
<th></th>
<th>Human</th>
<th>BB Rat</th>
<th>NOD Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of Onset</td>
<td>Any age *</td>
<td>50 - 120 days</td>
<td>70 - 210 days</td>
</tr>
<tr>
<td>Incidence</td>
<td>≈ 0.2%</td>
<td>80-90% †</td>
<td>70-80% †</td>
</tr>
<tr>
<td>Sex Susceptibility</td>
<td>Male ≥ Female</td>
<td>Male &gt; Female</td>
<td>Female &gt;&gt; Male</td>
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<tr>
<td>Hyperglycaemia</td>
<td>+++</td>
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<tr>
<td>Insulinopaenia</td>
<td>+++</td>
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<tr>
<td>Ketoacidosis</td>
<td>+++</td>
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<td>Insulitis</td>
<td>+</td>
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<td>Islet Atrophy</td>
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<tr>
<td>ICSA</td>
<td>Detectable</td>
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<tr>
<td>IAA</td>
<td>Detectable</td>
<td>Detectable?</td>
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<tr>
<td>64kDa Antibodies</td>
<td>Detectable</td>
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<tr>
<td>Lymphopaenia</td>
<td>±</td>
<td>+++</td>
<td>±</td>
</tr>
<tr>
<td>CD4⁺ T cells</td>
<td>Decreased</td>
<td>Decreased</td>
<td>Normal-Increased</td>
</tr>
<tr>
<td>CD8⁺ T cells</td>
<td>Increased</td>
<td>Decreased</td>
<td>Normal-Increased</td>
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<tr>
<td>CD4:CD8 ratio</td>
<td>Abnormal?</td>
<td>Abnormal</td>
<td>Normal</td>
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<td>la Expression</td>
<td>Increased</td>
<td>Increased</td>
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</tr>
<tr>
<td>Anti-β cell CTL</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Thyroiditis</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
</tr>
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* Most common before 20 years of age
† Varies according to subline or colony
Bueren, 1986), Keeshond Dog (Kramer et al., 1980), South African Hamster (Stuhlman et al., 1974) and Guinea Pig (MaxLang and Munger, 1976). These models, however, tend to have limited value in the study of human diabetes.

2.3.2 Induced Models

An alternative to searching for spontaneous diabetic syndromes in animals is to induce the disease experimentally. Chemical treatment or viral infection are the two predominant methods for inducing diabetes and have been used in a variety of animal species to provide models that are a useful adjunct to the spontaneous models. Although disease induced in this manner may not be as close to the human situation as disease arising spontaneously, these models have the advantage of not being constrained by numbers or availability of animals and are able to be introduced onto a range of genetic background by use of inbred animal strains.

i) Chemically Induced

Streptozotocin

Streptozotocin (STZ) is a broad range antibiotic and anti-tumour agent isolated from the bacterium Streptomyces achromogenes. Since the discovery of the diabetogenic properties of this chemical, STZ has become the most widely used agent for chemical induction of diabetes in animals.

The usual method of diabetes induction by STZ is to administer a single diabetogenic dose intraperitoneally (Paik et al., 1980) or multiple sub-diabetogenic doses administered intravenously or intraperitoneally on a
daily basis (Like and Rossini, 1976). Severe hyperglycaemia (greater than 40mM) is induced within 2 days of a single STZ injection, with B cell necrosis being detected microscopically 24 hours after treatment, but the islets remain virtually inflammation free (Brosky and Logothetopoulos, 1969). Hyperglycaemia develops more slowly after multi-dose STZ treatment (1 week or longer), and a mononuclear cell infiltration of the islets is present, the severity of which increases until reaching a peak 3 days after treatment (Bonnevie-Nielsen et al., 1981). The infiltration is characterized by infiltrating lymphocytes and macrophages in an environment of reduced B cell numbers and B cell necrosis.

Although an immunological pathogenesis for STZ-induced diabetes has been postulated, STZ has been shown to be toxic to lymphoid cells and islet cells in vitro (Gaulton et al., 1985; Charlton and Mandel, 1989). Thus it is possible that STZ is directly toxic to B cells in vivo or alters the antigenic structure of the B cell, making it a target for immune destruction.

**Other Chemically-Induced Models**

Other chemicals used to induce diabetes in animals include alloxan (2,4,5,6-tetra-oxo-hexa-hydro-pyrimidine), which acts to decrease B cell mass (McEvoy and Hegre, 1977; Chang and Diani, 1985), and various hormones such as glucagon, growth hormone, epinephrine, glucocorticoids, catecholamines and sex hormones which antagonize the actions of insulin (Mordes and Rossini, 1981; Chang and Diani, 1985). In addition, immunosuppressive drugs such as cyclophosphamide have been used to enhance the onset of diabetes in NOD mice (Harada and Makino, 1984; Charlton and Mandel, 1989).
ii) Virally Induced

Although a role for viruses in the aetiology of diabetes has long been postulated, it has never been established. Convincing evidence in favour of a link between viral infection and diabetes, however, is provided by the ability of certain viruses to induce diabetes in various animals.

Encephalomyocarditis (EMC) virus, an RNA virus isolated from the heart of a myocarditic pig, induces a diabetic syndrome similar to human IDDM in infected mice (Craighead and Steinke, 1971; Craighead, 1985). $\beta$ cell degranulation and decrease in islet IRI is associated with the detection of viral antigens in the pancreas of infected mice 3 days after inoculation (Craighead and Steinke, 1971; Notkins, 1977). Also seen at this stage is a mononuclear infiltration of islets, and this may suggest that disease onset is immune-mediated. Studies on immunosuppressed mice, however, have shown that this is not the case, but rather that the $\beta$ cell destruction is a result of viral aggression (Vialettes et al., 1980; Gould et al., 1985).

A diabetic syndrome can also be produced in mice by infection with Reovirus Types 1 and 3. These virus particles are able to infect $\beta$ cells and induce hyperglycaemia, abnormal glucose tolerance and hypoinsulinaemia (Onodera et al., 1978; Onodera et al., 1981). In addition, viral particles can be demonstrated in $\alpha$ and $\delta$ cells of mice infected with the type 1 strain. Both viruses induce polyendocrinopathy and autoantibodies to insulin (Onodera et al., 1981).

Other viruses capable of inducing diabetes include the Group B Coxsackie Virus, Venezuelan Encephalitis Virus (VEV), Cytomegalovirus (CMV),
Rubella Virus and Foot and Mouth Disease Virus (FMDV) (Steinke and Taylor, 1974; Notkins, 1977; Mordes and Rossini, 1981; Craighead, 1985). All are highly species specific RNA viruses and generally replicate in the β cell, causing functional alterations with eventual necrosis.

2.3.3 Transgenic Models

During recent years the use of transgenic animal models in the study of diabetes has become increasingly prevalent. Tissue-specific expression of foreign genes in islet β cells has become possible since the identification of the promoter/enhancer sequence for the insulin gene. Genes of interest linked to the insulin promoter can be expressed in high quantities in β cells only and a number of groups have used this approach to hyperexpress MHC class I or class II molecules on β cells of transgenic mice not usually prone to autoimmune disease. Allison et al. used this approach to hyperexpress mouse class I products (H-2K) on β cells. These mice did develop an insulin-dependent diabetic syndrome, although this was not associated with insulitis and was not inhibited by thymectomy, suggesting a non-immune mediated pathogenesis (Allison et al., 1988). This was later confirmed by experiments demonstrating an in vitro reduction in insulin production of excised H-2K transgenic pancreatic tissue (Mandel et al., 1991). Similarly, transgenic expression of class II (I-A) products on β cells in a normal strain of mouse and class II (I-E) products in mice deficient in I-E expression also produced a diabetic syndrome with no evidence of immune involvement (Sarvetnick et al., 1988; Lo et al., 1988). These experiments demonstrate that the extreme hyperexpression of MHC transgenic products under the insulin promoter is cytotoxic to β cells in vivo.
In contrast, transgenic expression of the lymphocyte activating cytokines interferon-γ (IFN-γ) or interleukin-2 (IL-2) on β cells produced an immune-mediated IDDM syndrome in all mice expressing the transgenes (Sarvetnick et al., 1990; Heath et al., 1992). Two groups have also examined transgenic expression of lymphocytic choriomeningitis virus (LCMV) glycoprotein on the surface of β cells (Oldstone et al., 1991; Ohashi et al., 1991). Both studies showed that mice were tolerant to the transgenic product during ontogeny, but this tolerance could be broken in adult animals by LCMV infection leading to an immune-mediated IDDM syndrome.

2.4 FACTORS INFLUENCING THE PATHOGENESIS OF IDDM

2.4.1 Genetic Factors

Since the observation that susceptibility to IDDM tends to be higher in families of IDDM patients than in the general population (see section 2.2.5), attention has focused on identifying genetic markers that may be implicated in the pathogenesis of the disease. The major histocompatibility complex (MHC) is a highly polymorphic cluster of genes that code for class I, II and III transplantation (histocompatibility) and immune response antigens. Numerous studies have provided evidence for a strong association between IDDM susceptibility and certain MHC class I and II haplotypes, both in humans and the spontaneous models of the disease.
i) Human

The MHC of humans is known as human leukocyte antigen (HLA) system and is found on the short arm of chromosome 6. Class I determinants of the HLA system are coded for by three separate regions termed HLA-A, B and C, while class II antigens are coded for by the HLA-D region, which is subdivided into 3 loci - DR, DQ and DP. As mentioned, molecules coded for by this region are highly polymorphic and these polymorphisms can be detected using alloantisera, monoclonal antibodies or alloreactive lymphocytes to allow identification, or typing, of allelic variants of HLA antigens within a population. More recently, molecular techniques such as restriction fragment length polymorphism (RFLP) or polymerase chain reaction (PCR) analysis have allowed polymorphisms at the genetic level to be identified (Baisch and Capra, 1992; Patel et al., 1992). Thus, for each individual, these alloantigenic variations can be identified and are given an identification code, for example HLA-A1, DR2, DQ12, DP3 etc. Polymorphisms at each locus identified by RFLP analysis or PCR are classified further as A or B to designate α or β chain and given a further set of numbers to identify the polymorphism, for example HLA-DQA1*0101.

Initial studies identified an association between the HLA-B antigens A8 and w15 and susceptibility to IDDM (Nerup et al., 1974; Cudworth and Woodrow, 1974). More recently it has become clear that MHC class I associations are not as strong as those of class II antigens, although a recent study has identified a decreased expression of class I proteins on peripheral cells from IDDM patients (Faustman et al., 1991). In particular, HLA-DR3 and DR4 have consistently shown a positive association with IDDM (Platz et al., 1981; Green, 1990). The presence of either DR3 or
DR4 confers an increased risk of developing IDDM, although this risk is greatly increased if both DR3 and DR4 are present (Platz et al., 1981).

Strong associations have also been found for DQ, but not DP, haplotypes (Baisch and Capra, 1992). An interesting association has been made between the DQB1 allele and IDDM (Todd et al., 1987; Baisch et al., 1992; Patel et al., 1992). Taking this further, Baisch and associates identified DQB1*0302 as conferring "dominant susceptibility" while DQB1*0602 provides protection (Baisch et al., 1990). Sequence analysis of the DQ class II β chain showed that DQB1 encodes a non-charged amino acid at position 57, whereas non-diabetogenic alleles at this locus encode a negatively charged aspartate (Asp) at this position (Todd, 1990). Position 57 of the DQ β chain has been predicted to have a position on the surface of the molecule where it is accessible to peptide and the T-cell antigen receptor (Brown et al., 1988). If the amino acid at position 57 is charged, such as Asp, this may allow an interaction with a neighbouring amino acid that inhibits peptide presentation by the MHC molecule and subsequent recognition by the T cell receptor, thus providing a protective effect. IDDM susceptibility cannot be entirely explained by the Asp57 relationship, however, and further work is needed to establish the molecular basis of associations with other markers such as DQα or other MHC haplotypes (Todd, 1990; Baisch et al., 1992).

In addition to MHC genes, polymorphisms in genes outside the MHC have been described. Millward and associates have identified a significant increase in the frequency of the 10.0;9.2 kb heterozygous phenotype of the Cβ chain of the T cell receptor in IDDM patients (Millward et al., 1987) and genes such as those coding for insulin and immunoglobulin heavy
chain that also appear linked to IDDM susceptibility (Todd and Bain, 1992).

ii) BB Rat

An association between IDDM susceptibility and the BB rat MHC complex has been well established. The rat MHC is termed RTI, the class I region being made up of RT1A, C and E loci and the class II region the RT1B and D loci (Gill et al., 1987). The BB rat expresses the RT1^u haplotype, and diabetes expression is independent of class I alleles but is dependent upon expression of at least one class II RT1^u allele (Colle et al., 1981) \textit{In vivo} anti-RT1D, but not RT1B, monoclonal antibody treatment reduces the incidence of diabetes, indicating that RT1D^u confers susceptibility to diabetes (Boitard et al., 1985). Molecular characterization of the RT1D β chains of BB rats and non-diabetes prone rat strains show that a serine residue is at position 57 in all strains, suggesting that Asp57 is not involved in resistance to IDDM in non-BB strain rats (Chao et al., 1989).

Cross-breeding studies between diabetes-prone and -resistant sublines of BB rats, both u/u homozygous at the MHC, consistently show that F1 generation hybrids are not susceptible to diabetes (Howard, 1983). This suggests that the original mutation that produced the DP subline of the BB strain occurred in a gene(s) outside the MHC, which in conjunction with the RT1^u background, predisposes to disease susceptibility. The role of this gene(s) in determining lymphopaenia and RT6^+ T cell depletion remains unclear.
iii) NOD Mouse

The MHC region of the mouse is termed H-2 and contains three loci encoding class I antigens termed H-2K, D and L and two loci encoding class II antigens termed I-A and I-E. NOD mice are H-2K^d and D^b, but differ from other mouse strains in that they do not express I-E antigens and have a unique I-A molecule, termed I-A^NOOD, in which the β chain Pro 56 and Asp57 are replaced by histidine and serine respectively (Hattori et al., 1986; Acha-Orbea and McDevitt, 1987). The development of transgenic technology has provided an ideal means by which to examine the role of I-A^NOOD and lack of I-E expression in diabetes susceptibility. Expression of I-E in transgenic NOD mice prevents the development of insulitis and diabetes in all mice (Nishimoto et al., 1987; Lund et al., 1990). It has been suggested that I-E expression during ontogeny deletes T cells expressing certain TCR Vβ chains (Reich et al., 1989), although the protective effect of this molecule remains unclear (Bohme et al., 1990).

In a second set of experiments, transgenic expression of I-A^K in NOD mice, which is Asp 57, markedly reduced insulitis and prevented onset of diabetes (Slattery et al., 1990) and a similar effect was seen if I-A^K Asp 57 was replaced by Ser 57 (Miyazaki et al., 1990). Transgenic expression of I-A^K with a proline at position 56 also inhibited disease onset (Lund et al., 1990). These experiments indicate that both lack of I-E expression and expression of the altered I-A^NOOD molecule contribute to diabetes susceptibility in NOD mice.

Backcross studies of NOD mice with the diabetes resistant NON strain have identified at least three additional genes, termed ldd-1, ldd-2 and ldd-3, that control the development of diabetes, two of which (ldd-2 and-
3) are not associated with the MHC (Prochazka et al., 1987). Wicker and associates, using NODxB10 backcrosses, also identified three independent genes controlling the onset of diabetes, only one of which was linked to the MHC, and also identified a single non-MHC linked gene controlling the initiation of insulitis (Wicker et al., 1987). In addition, as for human diabetes, defective cellular expression of class I molecules is also associated with NOD diabetes (Faustman et al., 1991).

2.4.2 Immunological Factors

The data outlined in the above sections provides strong evidence that the pathogenesis of IDDM, both in humans and the two major animal models, has an autoimmune aetiology based on the disruption of immunological tolerance to islet cell components. This evidence includes the detection of circulating antibodies to islet cell components, the presence of insulitis, in vitro reactivity of lymphoid cells to islet cell antigens and the association of IDDM with other endocrinopathies of autoimmune origin. A major focus of diabetes research has been to define the autoantigen(s) responsible for targeting the immune system to β cells and to characterize the events that allow this response to be initiated.

i) Autoantigens of IDDM

One of the remarkable features of the autoimmune response seen in IDDM is that it is highly β-cell specific. This implies that an autoantigenic molecule unique to β cells targets the immune system to these cells, whilst sparing other cell types within the islet. Of paramount importance for the development of any preventative strategy for IDDM is the identification and characterization of this β cell autoantigen(s). As
described in previous chapters, insulin itself has been the subject of interest for many years and although autoantibodies can be demonstrated, the role of these in disease induction is unclear and there have been no reports of T-cell reactivity. A number of other candidate molecules, including the 64kDa antigen, have been identified but as yet their pathogenicity has not been defined.

**Glutamic Acid Decarboxylase (GAD)**

As previously described, circulating antibodies of human patients and animal models immunoprecipitate a 64kDa islet specific molecule which seems to be a marker of disease onset. Identification of the target molecule of these antibodies may provide a starting point for identifying eventual T cell epitopes and consequently attention has focused on characterizing the 64kDa molecule.

Until 1990, the 64kDa antigen was known to consist of two subunits (α and β) of identical charge whose expression was restricted to the plasma membrane of islet β cells (Christie et al., 1990b; Baekkeskov et al., 1989). 125I surface labelling of islets followed by immunoprecipitation showed that the molecule was not a cell-surface component, and high glucose concentrations could stimulate increased expression of the molecule in vitro (Colman et al., 1987; Kampe et al., 1989).

The identity of the 64kDa molecule, however, was not established until late in 1990 when Baekkeskov and associates published a report showing immunoprecipitation of the 64kDa islet antigen by sera from patients with stiff man syndrome (SMS), a rare neurological disorder in which patients often have antibodies to islet cell cytoplasmic components and a high proportion of whom develop IDDM (Baekkeskov et al., 1990). The
principal autoantigen of SMS is the enzyme glutamic acid decarboxylase (GAD), which is involved in the synthesis of the inhibitory neurotransmitter γ-amino butyric acid (GABA) in the brain and also known to be present in small quantities in the islet. The fact that antibodies to GAD could immunoprecipitate an islet 64 kDa protein, and that anti-64kDa antibodies could precipitate GAD enzymic activity from islets and brain provided strong evidence that the 64kDa islet autoantigen is GAD. As a consequence of this finding many studies have attempted to characterize the structure and function of GAD in the islet and ascertain a role for this enzyme in the pathogenesis of IDDM.

Structure and Function of GAD

Prior to its association with IDDM, GAD was known to exist in bacteria and plants, with crystallized GAD from *Escherichia coli* being used to identify pyridoxal phosphate (vitamin B6) as a cofactor for enzyme activity (Strausbauch and Fischer, 1970; Sabo and Fischer, 1974). A role for GAD in the generation of GABA in the brain was postulated as early as 1950 (Roberts and Frankel, 1950). In 1973 mammalian GAD was purified from mouse brain and was recognized at this stage to be a dimeric molecule of 120 kDa, consisting of two subunits of 59 to 70 kDa (Wu et al., 1973). Using SDS-PAGE and immunoblotting, several groups reported that two different sized subunits of the GAD dimer could be identified - a smaller polypeptide of 59 to 60kDa and a larger polypeptide of 62 to 66kDa (Legay et al., 1986; Kaufman et al., 1986; Gottlieb et al., 1986; Chang and Gottlieb, 1988) and further evidence suggested that native brain GAD is a homodimer (Legay et al., 1987).

Thus, the conclusion was drawn that the two different polypeptides identified by SDS-PAGE and immunoblotting of GAD form two different
homodimeric GAD molecules in the brain. It was subsequently demonstrated that the small and large GAD polypeptides are encoded by two separate genes, and based on the calculated amino acid sequences it was recommended that they be designated GAD-65 and GAD-67 respectively (Erlander and Tobin, 1991; Bu et al., 1992). Multiple isoforms of brain GAD-67 have also been reported, differing in isoelectric points and hydrophobicities (Spink et al., 1987).

Apart from the brain, GAD activity has been detected in sites such as fallopian tubes, spermatozoa, kidney, liver, pituitary gland, thyroid gland, adrenal gland and notably in islets (Solimena and DeCamilli, 1991; Christie et al., 1992a). Isoforms of GAD expressed in sites other than brain or islets may be antigenically distinct and not targets for autoantibodies developing during the course of IDDM (Christie et al., 1992a). In the islet, GAD activity is confined to $\beta$-cells, and is concentrated at the surface of microvesicles (Reetz et al., 1991). Further analysis has shown that these vesicles are distinct from insulin secretory granules and GAD activity is also found in unique tubular cysternal complexes (Sorenson et al., 1991). GABAergic nerve cells have been found at the periphery of islets with intra-islet processes, and receptors for GABA have been detected on islet $\alpha$- and $\delta$-cells (Sorenson et al., 1991; Michalik and Erecinska, 1992). This data, in conjunction with evidence that the expression of GAD in islets is increased with an increased rate of insulin release (Bjork et al., 1992), suggests that GABA and GAD activity in the islet are involved with regulation of islet endocrine function.

Although both GAD-65 and GAD-67 have been reported to be expressed in islets, these reports tend to be somewhat conflicting and seem to depend on the species studied. Karlsen et al., identified two types of
cDNA coding for human GAD termed GAD-1 and GAD-2, and these encode GAD-67 and GAD-65 respectively (Karlsen et al., 1991). Northern blot analysis of mRNA from human and dog islets detected only a GAD-2 (GAD-65) transcript, while in brain tissue from a number of species both GAD-1 (GAD-67) and GAD-2 transcripts were detected. In contrast, Giorda et al. found expression of both GAD-65 and -67 in human islets, although GAD-65 seemed to be expressed at much higher levels (Giorda et al., 1991). In the rat, however, there seems to be general agreement that both isoforms of GAD are expressed at varying levels (Christgau et al., 1991; Michelsen et al., 1991; Karlsen et al., 1992). A possible explanation for the heterogeneity of islet GAD expression may relate to the handling and culture conditions of the islets prior to extraction, a point that needs to be addressed in further comparative studies.

Sequence analysis of GAD-65 and GAD-67 reveals that the brain and islet forms are identical, and there is a high degree of homology between GAD sequences of different mammalian species (Michelsen et al., 1991; Bu et al., 1992; Yamashita et al., 1992). In rat islets, GAD-67 seems to be hydrophilic and soluble with a pi of 6.9 to 7.1, while GAD-65 is a more abundant, amphiphilic protein of pi 6.7 (Christgau et al., 1991; Michelsen et al., 1991). In addition, GAD-65 appears to exist in 3 forms - hydrophobic and membrane bound, hydrophobic and soluble or hydrophilic and soluble - all of which can be separated into an α or β subunit by SDS-PAGE (Christgau et al., 1991). This heterogeneity in the structure of GAD may reflect the level of activity or function of GAD within the cell and has implications for the autoantigenic properties of this molecule in relation to IDDM.
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As yet there have been no reports of GAD autoantibodies in NOD mice or BB rats, presumably due to insufficient quantities of the antigen.

Since the identification of GAD as an autoantigen, its value as a predictive marker of IDDM onset has been of interest, but as yet is unclear. The association between anti-GAD antibodies and ICA is yet to be confirmed as Rowley et al., reported a weak association between the two, finding a higher proportion of GAD⁺ICA⁻ than GAD⁺ICA⁺ individuals among short and long term IDDM patients, whereas both Christie et al. and Martino et al. report a strong association between ICA, anti-GAD antibodies and the development of IDDM (Martino et al., 1991; Christie et al., 1992b). The "granular" pattern described for ICA staining (see section 2.2.4(i)) has been shown to be specific for GAD, but this pattern has a poor predictive value for disease onset, and studies have shown that it is not possible to remove ICA or decrease their titre when sera are pre-adsorbed with recombinant forms of the enzyme (Genovese et al., 1992; Timsit et al., 1992; DeAizpurua and Harrison, 1992).

As shown for the 64 kDa antigen, certain tryptic fragments of GAD increase the predictive value when compared to the intact molecule. In particular, a study of identical twins showed that the 37 and 40 kDa tryptic fragments had high predictive values, while antibodies to native GAD were present in genetically susceptible individuals who did not develop disease (Christie et al., 1992b). This study also identified a number of pre-diabetic individuals discordant for GAD and 37 kDa antibodies, raising the question of whether GAD accounts for all anti-64kDa reactivity. The prevalence of antibodies to GAD, however, is increased amongst individuals of the "high-risk" HLA-DR3 or DR4 genotypes (Serjeanston et al., 1992).
In contrast to antibody reactivity, there have been few reports describing T-cell reactivity to GAD. The first of these reports described the generation of T-cell lines specific for GAD from immunized DR-BB rats, which was not convincing as the authors failed to adequately define the purity of their antigen, which was then used to immunize animals and screen for positive T-cell clones (Diaz et al., 1992). In humans, PBL responses to GAD have been detected in 50 to 70 percent of newly diagnosed patients and a high percentage of ICA+ first degree relatives (Atkinson et al., 1992; Harrison et al., 1992b; DeAizpurua and Harrison, 1992). The most convincing evidence of a role for GAD in IDDM pathogenesis has come from two recent studies on tolerance induction in NOD mice (Kaufman et al., 1993; Tisch et al., 1993). Both studies showed that tolerization of GAD-reactive T cells by intravenous or intrathymic administration of GAD at an early age prevented the onset of IDDM in these mice. Furthermore, T-cell responses to GAD were detected during the normal course of disease at an early age, initially confined to a small region of the GAD molecule, but spreading to other GAD epitopes and eventually other β cell antigens with time.

Although there is strong evidence to show that GAD is an autoantigen in IDDM, it is still not clear whether this molecule is the primary target of β cell destruction, or whether the autoimmune response to this antigen is a result of prior β cell damage. The equivocal value of GAD as a predictor of disease onset, the difficulty in demonstrating T-cell reactivity to the molecule, the apparent lack of tissue specificity and the lack of data from animal models, particularly in relation to autoantibodies to GAD and disease induction by immunization, are some of the questions that need to be addressed to determine the pathogenic nature of this molecule.
Other Autoantigens

**Insulin:** Although other candidate autoantigens for IDDM have been suggested, none have been as extensively characterized as GAD and their aetiological role is far from clear. Insulin itself has been implicated as IAAs have been detected prior to the onset of disease in humans and animal models. With regard to T-cell reactivity, a low magnitude and frequency of PBL responses in preclinical or clinical IDDM patients (Harrison et al., 1991b) have been reported, while a study in NOD mice failed to demonstrate any insulin-specific T-cell reactivity (Hurtenbach and Maurer, 1989). More recent studies, however, have shown T cell reactivity to a variety of islet antigens including insulin in NOD mice (Kaufman et al., 1993; Tisch et al., 1993). Thus, insulin remains a potentially attractive autoantigen, although its role in disease pathogenesis remains unclear.

**38 kDa Autoantigen:** A 38 kDa insulin secretory granule antigen of the RINm5 insulinoma cell line cells has been shown to be the target of a T-cell clone generated from a recent-onset IDDM patient to crude RINm5 membranes (Roep et al., 1990). The clone responded strongly to 35-41 kDa RINm5 secretory granule membrane proteins, but also to neuroblastoma, pituitary and adrenal cell membranes preparations, raising questions about the tissue specificity of the antigen or the clone. This group also claimed to be able to generate T cell lines to this antigen from 80 percent of recent-onset patients (Roep et al., 1991). Pak et al. immunized Balb/c mice with human cytomegalovirus (CMV), a virus implicated in the pathogenesis of IDDM (Pak et al., 1988), and generated a panel of monoclonal antibodies, one of which reacted with islet cells (Pak et al., 1990). By immunoblotting, this antibody was shown to react with an islet cell protein of Mr 38kDa, suggesting that this may be a target
islet antigen of CMV-induced IDDM. In addition, a 38kDa band was observed in the initial human immunoprecipitation studies that identified the 64kDa antigen of islets (Baekkeskov et al., 1982) and Harrison and co-workers claim to have identified a 64kDa-antibody reactive clone from a human islet cDNA library producing a 38kDa protein that stimulates PBLs from preclinical IDDM subjects (Harrison, 1992).

58 kDa Autoantigen: Immunoblotting of NOD mouse sera against RINm5 extracts identified a 58kDa protein in 100 percent of diabetic NOD mice and in none of the controls (Boitard et al., 1992). Antibodies to this protein were also shown to cross-react with a 58kDa intermediate filament protein of neuroblastoma cells previously identified as peripherin. The 58kDa protein was also shown to share cross-reactive epitopes with MHC class II molecules, thus providing a means whereby islet 58kDa reactive T-cell clones may escape thymic deletion during ontogeny. Nevertheless, it remains to be determined whether peripherin of purified islet cells, as opposed to RINm5 insulinoma cells, is also autoantigenic.

Carboxypeptidase H: This is an enzyme of Mr 50 to 53kDa found within islet insulin secretory granules and involved in the conversion of proinsulin to insulin. It was identified by Castano et al., who screened a rat islet cDNA library with serum from a prediabetic IDDM subject and identified a positive clone whose DNA sequence corresponded to a fragment of the membrane form of carboxypeptidase H (Castano et al., 1991). Five of 20 ICA+ relatives and none of 14 controls reacted with a 52kDa protein of RINm5 extracts by immunoblotting, however, this study suffered from a lack of appropriate non-islet tissue controls, and only a positive reaction of one patient was shown.
Heat Shock Protein (HSP): Of considerable debate at present is the role of HSP's in the pathogenesis of diabetes in particular, and autoimmune disease in general. The interest in HSPs as autoantigens stemmed from the identification of a microbacterial HSP as an autoantigen in adjuvant arthritis (Feige and Cohen, 1991; Yang and Feige, 1991). Elias and co-workers first described an islet β cell antigen that was cross reactive with a 65 kDa HSP of Mycobacterium tuberculosis in NOD mice (Elias et al., 1990). These workers were able to show that at the onset of IDDM in NOD mice T lymphocytes reactive with HSP65 are detectable. With time, HSP65 antibodies and indeed the HSP65 antigen itself are demonstrable in the sera and as disease progresses, levels of antibodies, T-cell reactivity and antigen decline. Moreover, HSP65 could be used to either induce or vaccinate against diabetes depending on the mode of administration, and T cell clones raised against recombinant HSP65 could induce disease in recipient mice.

In a subsequent study, this group also reported that T-cell clones recognizing a peptide of HSP65 can transfer disease and can be used to immunize against disease (Elias et al., 1991). Jones et al. also described cross-reactivity between monoclonal antibodies to Mycobacterium tuberculosis HSP65, but not HSP from other sources, and the 64 kDa islet cell antigen by western blotting (Jones et al., 1990). Both these reports are refuted, however, by other studies showing no evidence for serological cross-reactivity between HSP65 and islet cell antigens (Kampe et al., 1990; Atkinson et al., 1991). Two reports have also suggested that an HSP60-like molecule is an islet cell antigen in IDDM. Brudzynski et al. demonstrated the presence of a mammalian HSP60 cross-reactive protein in the secretory granules and mitochondria of islet β cells, and a
subsequent study identified anti-HSP60 antibodies in the sera of diabetic and prediabetic mice (Brudzynski et al., 1992a; Brudzynski et al., 1992b).

ii) Cellular Mechanisms

Although abnormalities of peripheral lymphocyte populations and the association of IDDM with insulitis and cell-mediated autoimmune endocrinopathies provide circumstantial evidence for a role of cellular immune-mechanisms in the aetiology of IDDM, studies in animal models of the disease have been valuable in establishing a direct role for an autoimmune, cell-mediated pathogenesis for IDDM.

Direct evidence for the involvement of the immune system in the pathogenesis of IDDM comes from the following observations in both NOD mice and BB rats: 1) Treatment with anti-lymphocyte serum inhibits the onset of disease (Like et al., 1979; Maki et al., 1992); 2) Neonatal thymectomy inhibits disease onset and nude (athymic) animals do not develop disease (Like et al., 1982b; Ogawa et al., 1985); 3) Immunosuppressive agents such as cyclosporin are protective (Laupacis et al., 1983; Mori et al., 1986; Makino et al., 1986); 4) Disease can be transferred to naive recipients using lymphoid cells from diabetic donors (Koevary et al., 1983; Like et al., 1985; Wicker et al., 1986a and b) and; 5) The course of the disease can be inhibited by reconstituting susceptible animals with lymphoid cells derived from resistant donors (Burstein et al., 1989; Hutchings and Cooke, 1990).

Identification of the cell types involved in IDDM pathogenesis has also been facilitated by studies in animal models. Studies of adoptive transfer of disease in NOD mice generally show that in vitro deletion of either
CD4+ or CD8+ T cells inhibits the induction of disease, indicating a requirement for both of these cell types for the initiation of β cell destruction in this experimental system (Bendelac et al., 1987; Christianson et al., 1993). A number of studies have shown that in vivo depletion of CD4+ T cells in NOD mice prevents disease (Wang et al., 1987; Koike et al., 1987; Charlton and Mandel, 1988; Hutchings et al., 1992), however Charlton et al. also demonstrated an in vivo requirement for CD8+ T cells (Charlton et al., 1988). Analysis of cloned T cells isolated from infiltrated islets of NOD mice of different ages showed that the majority of clones isolated from young mice were CD4+, while CD8+ clones were readily isolated from older mice (Nagata and Yoon, 1992). In the BB rat, CD4+ T cells alone appear capable of transferring disease (Mordes et al., 1987; Metroz-Dayer et al., 1990) although in these studies the authors could not rule out interaction of these cells with other cell types. Other studies have shown that in vivo depletion of CD8+ T-cells (Like et al., 1986) or NK cells (Like et al., 1986; Jacobsen et al., 1988) inhibit the onset of diabetes in the BB rat.

Examination of the role of macrophages in the pathogenesis of IDDM in animal models has provided some interesting but apparently paradoxical observations. Macrophages have been shown to be present in islet infiltrations from a very early stage - even before the arrival of lymphocytes (Walker et al., 1988; Hanenberg et al., 1989). In addition, in vivo treatment of both BB rats and NOD mice with silica, a macrophage toxin, prevented the onset of diabetes (Charlton et al., 1988; Amano and Yoon, 1990). In vitro macrophage-mediated islet cell cytotoxicity is raised in BB rats at the onset of diabetes (Nagy et al., 1989) and the inhibition of macrophage migration into islets using a monoclonal
antibody to adhesion-promoting complement receptors inhibits development of disease in NOD mice (Hutchings and Cooke, 1990).

These observations imply that macrophages are essential for the development of insulitis and subsequent disease, possibly by mediating the initial β cell damage and/or recruiting lymphocytes into the islet. In contrast, the findings of several studies suggest that macrophages are capable of in vitro suppressive activity. Thus, two studies report dramatic improvement in the in vitro mitogen-induced proliferative responses and IL-2 production of spleen cells isolated from BB rats when depleted of adherent macrophage populations (Prud'homme et al., 1984; Woda and Padden, 1986) and a similar effect has been seen in the NOD mouse (Yokono et al., 1989).

In addition, studies have shown that vaccine-induced inhibition of insulitis and diabetes in NOD mice is mediated by activated macrophages. BCG (bacille Calmette-Guerin), a tuberculosis vaccine derived from attenuated Mycobacterium bovis and a nonspecific stimulator of cell mediated immunity, is capable of inhibiting diabetes onset in vaccinated NOD mice (Yagi et al., 1991a). Mice show reduced in vitro spleen-cell responsiveness to mitogens and alloantigens. Removal of adherent macrophages restores responsiveness and re-adding adherent cells restores suppression. In a subsequent study it was also shown that transfer of spleen-cell fractions enriched for macrophages from BCG-vaccinated mice to young female NOD mice inhibited the occurrence of diabetes (Yagi et al., 1991b). Metroz-Dayer et al. also reported improved in vitro mitogen activation and subsequent adoptive transfer of diabetes to young DP-BB rats by using macrophage-depleted spleen cells from diabetic donors (Metroz-Dayer et al., 1990).
Considered together, the above results suggest that the initial event in the pathogenesis of IDDM in these models is the infiltration of macrophages into the islet. CD4+ T cells follow, either as a result of β cell damage or recruitment by macrophages and then, in conjunction with CD8+ T cells, mediate β cell destruction. The role of suppressive macrophages is uncertain, but they may act to regulate this process either towards disease induction or suppression.

### iii) Cytokines

More recently there has been an increase in interest in the role of soluble mediators of immune function in the β cell destructive process. Cytokines are soluble immunoregulatory molecules produced by activated lymphocytes or monocytes that act as stimulatory or regulatory signals for both immune and non-immune cells. Although a wide variety of these molecules have been described that may act in the pathogenesis of IDDM, attention has focused on those shown to act directly or indirectly on the inflammatory response to β cells in humans and experimental systems.

The interleukin 1 (IL-1) family consists of three polypeptide signal molecules (IL-1α, IL-1β and IL-1 receptor antagonist) produced by activated mononuclear cells (Dinarello and Wolff, 1993). A number of studies have shown that IL-1, and in particular IL-1β, is able to inhibit islet-cell function and viability in vitro and in vivo, and is cytotoxic to β cells in vitro (Sandler et al., 1991; Welsh et al., 1991; Helqvist et al., 1991; Ling et al., 1993). This has led to the hypothesis that IL-1 may mediate β cell destruction in infiltrated islets (Mandrup-Poulsen et al., 1990). In contrast, long-term administration (5 times per week for 22 weeks) of IL-1α was able to inhibit spontaneous insulitis and diabetes in
NOD mice and IL-1α treatment of recipients inhibited the adoptive transfer of disease by CD4+ cells isolated from diabetic donors to young NOD mice (Formby et al., 1992b). In BB rats, short term administration (twice daily for 3 weeks) of IL-1 receptor antagonist delayed the onset of diabetes (Dinarello and Wolff, 1993).

Tumour necrosis factor alpha (TNFα) is another macrophage derived cytokine that has been implicated in the pathogenesis of IDDM. Long-term administration of TNFα to NOD mice or BB rats prevents the onset of diabetes (Jacob et al., 1990; Satoh et al., 1990) while diabetic and pre-diabetic NOD mice have been shown to have increased circulating levels of this cytokine (Barel et al., 1992). Interferon gamma (IFNγ), produced by activated T cells, has also been implicated as BB rats treated with poly I:C, a stimulator of IFNγ production, accelerates diabetes in BB rats (Ewel et al., 1992) while *in vivo* treatment with IFNγ monoclonal antibodies prevents disease onset (Nicoletti et al., 1990). In human patients, a defect in the synthesis of another activated lymphocyte-derived cytokine, interleukin-2 (IL-2), has been observed (Zier et al., 1984; Kaye et al., 1986).

It is evident from the above results that although there appears to be a role for cytokines in the pathogenesis of IDDM, the mechanism is far from clear and will probably involve a network of complex interactions. Nevertheless, an understanding of these interactions will ultimately be essential for eventually determining the pathogenic mechanisms of this disease.
2.4.3 Environmental Factors

Although a strong genetic influence has been established for susceptibility to IDDM, genetics alone cannot account for a number of observations in humans and animal models. The observations that 1) a number of individuals with susceptible MHC haplotypes do not develop disease 2) the concordance rate for IDDM in identical twins is less than 50 percent 3) incidence rates vary according to season and geographical location and 4) the variable incidence rates seen between colonies of inbred BB rats and NOD mice, together strongly suggest a role for non-genetically determined environmental factors in the pathogenesis of IDDM.

i) Infectious Agents

A role for viruses in the aetiology of diabetes has long been suspected, but not confirmed. Association between diabetes and mumps, Rubella, hepatitis, coxsackie and influenza viruses, cytomegalovirus and enterovirus have been proposed, but little epidemiological evidence has been provided in support of the association (Maugh, 1975). A Coxsackie B4-like virus has been isolated from the pancreas of a patient with diabetic ketoacidosis (Yoon et al., 1979). Anti-viral antibodies were present in the serum and viral antigens were demonstrated in β-cells.

As previously described (2.3.2), viral infection has been associated with diabetes in the BB rat and NOD mouse, and experimental viral infection can induce diabetes in normal animals. In contrast, however, are studies showing that infection of NOD mice with mouse hepatitis virus or lymphocytic choriomeningitis virus reduced the incidence of disease (Oldstone, 1988; Wilberz et al., 1991).
Further evidence of a role for infectious agents comes from studies in the BB rat. Like et al. reported that DP rats raised under viral antibody-free (VAF) conditions showed increased frequency and accelerated onset of diabetes (Like et al., 1991) and an outbreak of spontaneous diabetes in the DR-BB subline was associated with the onset of viral infection in these animals (Thomas et al., 1991). This is in contrast to a study by Rossini and associates, who showed no difference in the incidence levels of disease between DP-BB rats reared under conventional or gnotobiotic conditions (Rossini et al., 1979). Ellerman and Like examined the possibility that T-cell stimulatory properties of Staphylococcal enterotoxin (SE), produced during Staphylococcal aureus infections, may be involved in the activation of quiescent self reactive T-cells (Ellerman and Like, 1992). In vitro SE activation of spleen cells from diabetic BB was effective in stimulating diabetogenic effector cells capable of transferring disease to young DP animals. This raises the possibility that T-cell stimulatory microorganisms may play a role in IDDM pathogenesis.

ii) Diet

The relationship between IDDM and diet in humans is difficult to study due to the highly varied nature of the human diet. Studies in both BB rats and NOD mice, however, suggest that modifications of one or more components of the diet - for example protein, carbohydrate or fat content - are able to modify the incidence of disease (Helgason et al., 1982; Elliott and Martin, 1984; Scott et al., 1985; Atkinson et al., 1988; Elliott et al., 1988). In contrast, other studies in the BB rat have indicated that neither the incidence nor severity of the diabetes is influenced by dietary modification (Rossini et al., 1981). The subject, therefore, still remains controversial, but in the numerous studies in favour of a dietary
component, protein content and composition appear to be the most influential factors in disease induction.

Recently attention has focused on cow's milk proteins as being potential triggers of IDDM. Breast-feeding has been observed to have a protective effect (Mayer et al., 1988; Kostraba et al., 1992) and a number of recent studies have reported a high incidence of antibodies to cow's milk proteins in patients with diabetes mellitus. Dahlquist et al. found a positive correlation between antibodies to β-lactoglobulin and risk for developing early-age onset IDDM (Dahlquist et al., 1992). Similarly, Karjalainen and associates identified elevated levels of antibodies to bovine serum albumin (BSA) in 100 percent of newly-diagnosed patients (Karjalainen et al., 1992a). Furthermore, these workers identified a peptide sequence of BSA that differed from the amino acid sequence of human, rat and mouse albumins and showed that BSA antibodies from diabetic patients were specific for this peptide (Karjalainen et al., 1992b). Antibodies to BSA have also been demonstrated in BB rats and NOD mice, and these antibodies have been shown to cross react with a 69 kDa islet-cell protein termed p69 (Glerum et al., 1989; Beppu et al., 1987). Thus the authors argue that the triggering event of IDDM could be the cross-reaction of antibodies to a peptide of BSA with an islet-cell protein in genetically susceptible individuals.

### 2.5 CONCLUSIONS

From the above studies it is clear that IDDM is a disease characterized by profound disregulation of carbohydrate metabolism resulting from the immune-mediated destruction of insulin-secreting β-cells. The destructive
phase of the disease is a cell-mediated event, although the disease appears to have a long prodromal period during which autoantibodies to a variety of islet and non-islet components are generated. Of these, ICA and 64kDa antibodies are the best-characterized and together provide the best predictive markers of disease progression.

Several animal models of IDDM are available and of these, the BB rat and the NOD mouse have been extensively characterized. In both these models, disease arises spontaneously and while closely approximating the human condition, they are not identical. While several models of IDDM are available in which the disease is actively induced, they all require some form of chemical treatment or viral infection, often making interpretation of the results difficult.

Studies in both humans and animals suggest an important role for genetic factors in the pathogenesis of the disease, particularly in relation to products of the class II region of the MHC. Nevertheless, susceptibility to IDDM appears to be a multi-factorial process, involving both genetic and environmental factors. Identification of these environmental factors and the way they influence the pathogenesis of IDDM, in conjunction with genetic studies, could ultimately allow development of effective preventative regimes.
CHAPTER 3.0

CLINICAL AND DYNAMIC FEATURES OF THE Tx-X

INDUCED DIABETIC SYNDROME
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3.1 INTRODUCTION

Experimental animal models of human disease allow approaches to the investigation of factors that contribute to disease initiation and pathogenesis that are often not possible in humans. Animal models of human IDDM have provided much useful information, particularly with regard to the role of the immune system in disease pathogenesis.

IDDM may develop in animals of particular genetic background spontaneously (that is, genetically determined) or may be induced by various chemical or viral induction protocols that disrupt immunological tolerance to islet-cell antigens (reviewed in the previous chapter). Although it can be argued that animal models of IDDM in which disease develops spontaneously more closely resemble the disease of humans, artificially induced models have the advantage of being more amenable to experimental manipulation, particularly with regard to the absence of genetic constraints and the availability of genetically identical, normal animals for control purposes. To date however, no well-characterized induced model of human IDDM has been available that does not rely on chemical or infectious agents for disease expression. The availability of such a model would offer an adjunct to the currently available spontaneous models for the study of disease pathogenesis, while avoiding the complications arising from the directly pathogenic effects of these agents.

A combination of thymectomy and irradiation (Tx-X), a procedure known to alter immune function and regulation, was first described by Penhale and associates as a means of inducing lymphocytic thyroiditis in rats (Penhale et al., 1973). The induced thyroiditis was characterized by
infiltration of lymphocytes and macrophages into the thyroid gland, and studies by this group suggested that the disease was immune-mediated and not the result of direct thyroid tissue damage *per se* (Penhale et al., 1973; Penhale et al., 1976).

The Tx-X model has subsequently proven readily amenable to procedures capable of modifying expression of disease, with previous studies showing that treatment with anti-lymphocyte serum or reconstitution with normal syngeneic lymphoid cells inhibited thyroiditis onset (Penhale et al., 1975; Penhale et al., 1976), while thyroiditis could be transferred to naive recipients using activated spleen cells from thyroiditic donors (Penhale and Stumbles, unpublished observations). These observations indicated a causative role for the immune system in the pathogenesis of the disease and argued strongly in favour of autoimmune origin for Tx-X-induced thyroiditis.

Originally, the Tx-X model was developed in Wistar strain rats of conventional hygienic background. More recently, however, it was observed that changing to PVG strain rats of specific pathogen free (SPF) background induced an acute-onset and rapidly fatal wasting syndrome in addition to the previously observed thyroiditis. The ability to control this wasting syndrome by exogenous insulin therapy suggested that this disease was analogous to human IDDM

This chapter describes the clinical and pathological features of this diabetic syndrome consistently observed in SPF PVG rats following the Tx-X procedure. The syndrome appeared closely analogous to clinical IDDM in man and the model thus has potential value for studies of diabetes initiation and pathogenesis.
3.2 MATERIALS AND METHODS

3.2.1 Animals

Unless otherwise stated, all experiments utilized female PVG (RT1^c) rats reared under SPF conditions obtained from the Animal Resources Centre, Murdoch, Western Australia and housed under non-barrier conditions in the University animal house. Rats were maintained in groups of 3-5 per cage and fed on standard pelleted rat feed with water provided ad libitum. Female WAG (RT1^u), Lou/m (RT1^u), SHR (RT1^k), DA (RT1^a) and F344 (RT1^l) strain rats were obtained from the same source and housed under the same conditions. To examine the influence of environmental background on disease inducement, inbred PVG rats were bred under non-SPF conditions (conventional) and maintained in a separate room. All PVG animals, whether SPF or conventional, were derived from the same parental stock. Twenty-one day old F1 generation RT1^{c/u} hybrid rats were derived from SPF PVG (RT1^c) female/WF (RT1^u) male matings.

3.2.2 Tx-X Protocol

This was performed basically as described by Penhale et al., 1973. Rats were thymectomized on arrival from the SPF unit under sodium pentobarbitone anaesthesia then given 4 fortnightly doses of low-dose (2.5 Gray) whole-body gamma irradiation using a Cobalt-60 source. Rats were treated in groups of 20 to 30 at one time, housed in groups of 3 to 5 and maintained for 8 to 10 weeks after final irradiation, during which time diabetes developed spontaneously (Figure 3.1). All Tx-X rats were retrospectively checked at necropsy for the efficacy of thymectomy and
Figure 3.1. Protocol for the induction of diabetes by thymectomy and irradiation. PVG rats were weaned at 3 weeks of age, thymectomized (Tx) during the following week then given four fortnightly doses of whole-body gamma irradiation (X). During the following 10 weeks, disease developed spontaneously.
data from any animal with thymic remnants excluded from the study (less than 1 percent of all animals used in this study).

3.2.3 Disease Monitoring

During the 8 to 10 week development period following final irradiation animals were examined daily for early signs of diabetes, detectable by a rapid loss of weight and behavioural changes. Affected animals were then tested for hyperglycaemia by Glucostix® and a Glucometer® analysis (Bayer Diagnostics, Mulgrave, Australia) using caudal vein blood samples taken under ether anaesthesia, and classified as diabetic on the basis of a blood glucose reading equal to or greater than 13.5 mM. Clinically diabetic animals were treated daily with subcutaneous doses of human insulin (1.5 units each of Protaphane® and Actrapid®; CSL Novo, Melbourne, Australia). Animals were necropsied shortly after onset of clinical signs, or at the end of the 8 to 10 week period if clinical disease did not develop, and plasma samples prepared for enzymatic glucose determination (Cobas Mira biochemical analyzer) and pancreata and thyroids taken for histopathology.

In some cases glucose tolerance tests were performed to establish irregularities in glucose metabolism. Blood samples for glucose determination were taken from non-fasted animals prior to the intraperitoneal (IP) injection of 2 mg per gram body weight of glucose dissolved in 0.15 M phosphate buffered saline, pH 7.3 (PBS). Blood glucose levels were then determined at 30, 60 and 120 minutes intervals.
3.2.4 Histopathology

Histopathological change was assessed on formalin-fixed, haematoxylin and eosin (H&E) stained pancreas sections. Islet lesions were classified as either i) *diffuse atrophy* if all islets were affected ii) *focal atrophy* if only a proportion of islets were atrophic and/or iii) *insulitis* if lymphocytic infiltration of islets was observed. Sections were assessed without prior knowledge of clinical status.

Thyroid glands were taken at the same time and assessed for thyroiditis after formalin-fixation and H&E staining. Lesions were graded on a scale of 1 (mild) to 4 (severe) according to the severity of the infiltration and degree of follicular destruction.

3.2.5 Immunofluorescence

Formalin-fixed sections of rat pancreas were examined for the presence and distribution of α (glucagon-synthesizing) and β (insulin-synthesizing) cells by indirect immunofluorescence. β cells were stained using a mouse anti-human insulin monoclonal antibody (Amersham Int., Amersham, U.K.) undiluted for 18 hours at 4°C, followed by a sheep anti mouse IgG-fluorescein isothiocyanate (FITC) conjugated antiserum (Silenus Laboratories, Melbourne, Australia) diluted 1:40 for 1 hour at room temperature. α cells were stained in a similar manner using a rabbit anti-human glucagon polyclonal antibody (Amersham Int., Amersham, U.K.) followed by a goat anti-rabbit IgG-FITC conjugated antiserum (Miles Yeda, Israel).
3.2.6 Plasma Insulin and Lipid Analysis

Plasma immune-reactive insulin (IRI) and lipid levels were analyzed in a proportion of samples from acute diabetic animals (prior to insulin treatment) or from normal (non-Tx-X) rats. IRI radioimmunoassays were performed by the Department of Medicine, Royal Postgraduate Medical School, London, U.K. and acetoacetate and β-hydroxybutyrate levels determined enzymatically by the Department of Biochemistry, School of Veterinary Studies, Murdoch University.

3.2.7 Tests for Pathogens

Serum samples from parent breeding stock rats were tested routinely (3 month intervals, n=60) by the Department of Agriculture, South Australia for antibodies to the following common rodent pathogens: Mycoplasma pulmonis, murine hepatitis/rat coronavirus, murine cytomegalovirus, minute virus of mouse, pneumonia virus of mouse, Reovirus type 3, adenovirus, lymphocytic choriomeningitis, mouse encephalomyelitis, encephalo- myocarditis, Sendai, Kilham's rat, Toolan HI and Hantaan viruses. A small group of parent breeding stock (n=6) were randomly selected every 2 months and pharyngeal and caecal samples examined for the presence of a range of recognized bacterial and parasitic pathogens (Pasteurella sp., Yersinia sp., Bordetella sp., Streptococcus sp., Listeria sp., Salmonella sp., Streptobacillus moniliformis, Hexamita and pinworm). In addition, a smaller number of samples from SPF or conventional Tx-X rats were also tested. All samples were found to be negative on all occasions except one Tx-X, SPF-derived, non-diabetic sample which was weakly positive for antibody to M. pulmonis. Larval
stages of the liver parasite *Taenia taeniaeformis* were occasionally isolated from conventionally-derived animals.

### 3.2.8 Statistical Analyses

Comparison of incidence rates was by chi-squared analysis of 2 x 2 contingency tables using the Yates' correction for sample numbers greater than 50 or Fishers' Exact Test for those less than 50 (Langley, 1971). Mean values were compared using Student's t-test for unpaired samples.

### 3.3 RESULTS

#### 3.3.1 Characterization of the Diabetic Syndrome

i) Clinical Features

Onset of acute diabetes could be recognized visually by a rapid loss of body condition (greater than 5 grams total body weight in less than 24 hours) and was confirmed by a concurrent increase in blood glucose levels. Blood glucose levels rose rapidly over a period of 2 to 3 days in untreated animals to levels of 50 to 60mM in some cases (Figure 3.2). Initiation of parenteral insulin therapy at onset of these symptoms was effective in stabilizing blood glucose levels and controlling weight loss, thus restoring body weight and condition in the majority of cases (Figure 3.3). Once stabilized, diabetic rats could be maintained indefinitely on daily insulin therapy, whereas delay or withdrawal of insulin resulted in
Figure 3.2. Blood glucose levels of Tx-X rats following final irradiation. Rats were bled weekly for 6 weeks following final irradiation and blood glucose levels determined. Animals not developing diabetes (squares) maintained relatively constant blood glucose levels throughout this period (n=20), while acute diabetic animals (circles) showed rapid increases in glucose levels at the onset of disease if not treated with insulin.
Figure 3.3. Body-weight profiles of diabetic rats prior to and following insulin therapy.
further loss of condition and hyperglycaemia, leading to death within 1 to 3 days. IP glucose tolerance tests performed on newly-diagnosed diabetic rats showed a steady increase in blood glucose levels from initially elevated levels to levels greater than 30 mM after 120 minutes in most cases, indicating a profound disruption in glucose metabolism in these animals (Figure 3.4).

A comparison of the plasma chemistry of acute diabetic Tx-X rats and normal rats is shown in Table 3.1. Diabetic animals had markedly raised levels of blood glucose, acetoacetate and β-hydroxybutyrate \((P < 0.001)\) and correspondingly depressed circulating insulin levels in plasma samples taken prior to necropsy compared to age-matched controls \((P < 0.001)\). Thus profound disturbances in glucose regulation and compensatory changes in fat metabolism were observed in diabetic rats.

ii) Histopathology

Pancreatic sections taken from acute diabetic rats shortly after onset of clinical signs revealed severe lesions usually confined to the islets (Figure 3.5). The degree of islet change generally reflected the clinical condition of the animal, ranging from diffuse atrophy in acute diabetic animals (Figure 3.5a) to varying degrees of insulitis and focal atrophy in chronic and non-diabetic Tx-X rats (Figure 3.5b).

Based on the above clinical symptoms and/or islet histopathology, Tx-X rats could be classified into one of four groups: 1) - acute diabetes. These rats developed full clinical symptoms and showed islet lesions (insulitis or atrophy) at necropsy; 2) - chronic diabetes. Identified
Figure 3.4. IP glucose tolerance tests of acute diabetic (circles) and clinically non-diabetic (squares; n=5) Tx-X rats. Rats were starved overnight and challenged IP with glucose at time 0, then blood glucose readings taken at 30, 60 and 120 minute intervals.
Table 3.1. Plasma chemistry of acute diabetic and normal PVG rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>n</th>
<th>Acute Diabetes *</th>
<th>Normal (non-Tx-X)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mM)</td>
<td>7</td>
<td>50.9 ± 1.9</td>
<td>10.8 ± 0.4</td>
</tr>
<tr>
<td>Insulin (pM) †</td>
<td>7</td>
<td>9.4 ± 3.3</td>
<td>49.9 ± 5.8</td>
</tr>
<tr>
<td>Acetoacetate (mM)</td>
<td>10</td>
<td>0.97 ± 0.3</td>
<td>0.02</td>
</tr>
<tr>
<td>β-hydroxybutyrate (mM)</td>
<td>10</td>
<td>9.11 ± 1.5</td>
<td>0.37 ± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± SEM

* Hyperglycaemia with insulin-dependence. Samples taken at onset of clinical signs
† Detection limit of assay = 5pM

P values less than 0.001 for all comparisons
Figure 3.5. Islet histopathology of Tx-X rats. Pancreas sections taken from a diabetic rat at disease-onset (a) and a non-diabetic rat at the end of the induction period (b) were formalin-fixed and stained by H&E (x250).

a) Diffuse atrophy of an acute diabetic rat - note loss of islet cell mass.

b) Insulitis of a non-diabetic Tx-X rat - note infiltration of mononuclear cells
Figure 3.5

c) Normal Tx-X rat islets (H&E; x250).
retrospectively on the basis of islet lesions and hyperglycaemia detected in samples taken prior to necropsy. These animals maintained normal body weight and condition throughout the observation period and did not become insulin-dependent; 3) normoglycaemic (non-diabetic) Tx-X rats with islet lesions at necropsy; 4) normoglycaemic Tx-X rats with normal islets at necropsy.

The frequency with which islet lesions (atrophy and/or insulitis) were observed in the above-described groups of Tx-X rats is summarized in Table 3.2. Diffuse atrophy was confined to the majority (75 percent) of acute diabetic animals, consistent with major impairment of $\beta$ cell function within this group ($P < 0.001$). Focal atrophy, usually together with insulitis, was also common in acute diabetic rats (22 percent; $P < 0.001$ compared with normals) and also in chronic diabetic rats (37 percent). Insulitis was rarely seen in animals with acute disease, but this was the most frequent lesion of chronic and non-diabetic Tx-X rats. Islet atrophy or insulitis were never observed in control unmanipulated PVG animals ($n = 25$).

In addition, a high frequency of lymphocytic thyroiditis was observed in Tx-X rats. This lesion was characterized by mild to severe infiltration of lymphoid cells into the gland, resulting in partial to total loss of follicular integrity (Figure 3.6). As shown in Table 3.2, thyroiditis was observed with high frequency in chronic and non-diabetic Tx-X rats (63 percent and 56 percent respectively) but at lower levels in acute diabetic animals (36 percent). Thyroiditis occurred with an overall incidence of 49 percent, but showed no apparent correlation with acute diabetes.
Table 3.2. Frequency of autoimmune lesions in Tx-X rats.

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Diabetic</th>
<th>Non-diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acute</td>
<td>Chronic</td>
</tr>
<tr>
<td>$n$</td>
<td>$n$ (%)</td>
<td>$n$ (%)</td>
</tr>
<tr>
<td>76</td>
<td>8</td>
<td>85</td>
</tr>
<tr>
<td>Diffuse Atrophy</td>
<td>57 (75)</td>
<td>0</td>
</tr>
<tr>
<td>Focal Atrophy</td>
<td>17 (22)</td>
<td>3 (37)</td>
</tr>
<tr>
<td>Insulitis</td>
<td>2 (3)</td>
<td>5 (63)</td>
</tr>
<tr>
<td>Thyroiditis</td>
<td>27 (36)</td>
<td>5 (63)</td>
</tr>
</tbody>
</table>

* Hyperglycaemia with insulin-dependence
† Hyperglycaemia without insulin-dependence
§ Normoglycaemic
$\parallel$ $P < 0.001$ compared with non-diabetic rats
$#$ $P < 0.01$ compared with non-diabetic rats
Figure 3.6. Lymphocytic thyroiditis of a Tx-X rat. Note heavy infiltration of mononuclear cells and destruction of thyroid follicles (H&E stain; x250).
The incidence of islet lesions within groups of Tx-X rats is shown in Figure 3.7 (open bars). In some groups (for example, Group 18), 100 percent of animals with islet lesions developed acute disease, whereas for other groups (for example, Group 10) the proportion was less than 50 percent. Overall, incidence of islet lesions in Tx-X rats (whether acute diabetic or otherwise) ranged from 17 percent to 75 percent between groups, with an overall mean of 47 percent for all Tx-X rats in this study (n = 392). Acute diabetes or islet lesions were not detected in normal, unmanipulated PVG rats maintained over the same period (n=25).

In order to determine the selectivity of the pathogenic process within islets during the course of the disease, sections of atrophied or infiltrated islets were stained by immunofluorescence to demonstrate the presence and distribution of α (glucagon-synthesizing) and β (insulin-synthesizing) cell types (Figure 3.8). Atrophic islets failed to stain with the insulin antibody, indicating that a complete loss of β cells was characteristic of end-stage atrophy (Figures 3.8a and e). In contrast, α cells were readily detected in atrophic islets and had become the predominant cell type, now in a central location (Figures 3.8b and f). In infiltrated islets there was an obvious loss of the normal structure, where although both α and β cells could still be detected in these islets, the β cells were dispersed throughout the islet rather than occupying their usual central location (Figures 3.8c and d). This data illustrates that β cells are the primary target for immunological destruction in islets of acute diabetic Tx-X rats.

iii) Onset and Incidence

Onset of the acute diabetic syndrome in Tx-X rats occurred at all stages of the 10 week development period, however a peak period of
Figure 3.7. Incidence of islet lesions (open bars) and acute diabetes (hatched bars) in sequential groups of Tx-X rats established at monthly intervals. Pancreas samples for histology were taken at the end of the induction period for non-diabetic animals or at time of disease onset for acute diabetic rats and assessed for islet lesions (insulitis or atrophy). Hatched bars within open bars indicate the proportion of animals with islet lesions that developed acute diabetes for each Tx-X group.
Figure 3.8. Immunofluorescent staining of Tx-X rat islets for α (glucagon) and β (insulin) cell types. Sections of pancreas showing islet atrophy or insulitis were stained with an antibody to insulin or glucagon, labelled with a FITC-conjugated antiserum and photographed under a fluorescent microscope (x250 magnification).

a) Atrophic islet stained for insulin showing complete loss of β cells.
b) Atrophic islet stained for glucagon showing redistribution of α cells.
Figure 3.8.

c) Infiltrated islet stained for insulin showing dispersed $\beta$ cells.

d) Infiltrated islet stained for glucagon showing dispersed $\alpha$ cells.
Figure 3.8.

e) Normal islet stained for insulin showing central location of \( \beta \) cells.

f) Normal islet stained for glucagon showing peripheral location of \( \alpha \) cells.
susceptibility was observed at 4 weeks after final irradiation, with onset of disease being rare before week 3 or after week 8 (Figure 3.9). The incidence of acute diabetes in successive Tx-X groups established at one-month intervals was previously shown in Figure 3.5 (shaded bars). Although acute diabetes occurred regularly, there were considerable variations in incidence levels, ranging from 11 to 62 percent between groups. Overall, acute diabetes occurred with a mean incidence of 34 percent for all Tx-X rats established over this period (392 rats in 19 separate groups).

3.3.2 Factors Affecting Disease Expression

i) Gender

In both humans and BB rats, diabetes occurs with close to an equal frequency in males and females. In contrast, female NOD mice are markedly more susceptible to disease. In order to investigate the influence of gender on the susceptibility of PVG rats to Tx-X-induced IDDM, groups of male and female SPF-derived rats were established. Mean levels of acute diabetes were slightly higher in males when compared with females, but this difference was not statistically significant (Table 3.3). Incidence of islet lesions, however, were significantly raised in male animals \( (P < 0.05) \). Disease onset was similar in both males and females, with all animals becoming diabetic between 10 and 60 days post irradiation and onset was at a similar rate in both groups during this period (Figure 3.10).
Figure 3.9. Incidence of acute diabetes in Tx-X rats following final irradiation.
Table 3.3. Incidence of diabetes and islet lesions Tx-X rats of SPF or conventional origin.

<table>
<thead>
<tr>
<th>Environmental Background</th>
<th>Diabetes</th>
<th>Islet Lesions §</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Acute *</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>n (%)</td>
</tr>
<tr>
<td>SPF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>68</td>
<td>20 (29)</td>
</tr>
<tr>
<td>Male</td>
<td>58</td>
<td>21 (36)</td>
</tr>
<tr>
<td>Total</td>
<td>126</td>
<td>41 (33)</td>
</tr>
<tr>
<td>Conventional</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>Male</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>51</td>
<td>0</td>
</tr>
</tbody>
</table>

* Hyperglycaemic with insulin-dependence
† Hyperglycaemic without insulin-dependence
§ Islet atrophy or insulitis in both diabetic and non-diabetic rats
‖ P < 0.05 compared with SPF female value
¶ P < 0.005 compared with SPF value
# P < 0.001 compared with SPF value
‡ P < 0.025 compared with SPF value
Figure 3.10. Comparison of the cumulative incidence of acute diabetes in male and female Tx-X rats following final irradiation.
ii) Environmental

Environmental factors have long been postulated to play a role in the pathogenesis of autoimmune disease in general and diabetes in particular, and various microbial agents have been shown to modify the course of disease in both BB rats and NOD mice (see section 2.4.3). In order to assess the possible influence of environmental factors in the pathogenesis of Tx-X-IDDM, disease expression was compared in genetically identical rats derived under clean (SPF) or conventional hygienic conditions (see Table 3.3). Conventionally-bred rats showed a strikingly reduced susceptibility to diabetes induction, with no conventionally-derived animals developing diabetes (n=51) compared with 41/126 (33 percent) of SPF controls \( (P < 0.001) \). A significant (but not complete) reduction in the incidence of islet lesions was also observed in conventional as compared with control SPF animals \( (P < 0.025) \).

For thyroid lesions, conventionally-reared female animals remained susceptible to thyroiditis, with 9/16 (56 percent) of animals developing disease. In contrast, conventionally-reared male animals appeared less susceptible, with only 3/17 (18 percent) of rats developing thyroid lesions.

iii) Genetic

Susceptibility to diabetes is known to have a strong genetic component as shown by the familial association of disease, and has been linked to various MHC class II genetic markers such as HLA-DR3 and DR4 in humans, \( \text{I-A}^{\text{NOD}} \) in the NOD mouse and \( \text{RT1}^{\text{U}} \) in the BB rat (see sections 2.2.5 and 2.4.1). To examine the influence of MHC haplotype on
Tx-X induced IDDM, RT1^u/c cross-bred animals and a number of inbred SPF-derived rat strains of differing RT1 background were examined.

F1 generation PVG (RT1^c)/WF (RT1^u) crosses were established primarily to examine the influence of RT1^u superimposition on the PVG genetic background (Table 3.4). Male crossbred animals showed no significant difference in the frequency of diabetes or islet lesions when compared with male or female inbred animals, but the frequency of islet lesions was raised in this group when compared to female crossbred animals (53 and 19 percent respectively; \( P < 0.05 \)). Acute diabetes occurred with a significantly higher frequency in female crossbred animals than in female inbred animals (50 and 29 percent respectively; \( P < 0.05 \)), however no differences were observed between female and male crossbred animals. The incidence of islet lesions was lower in female crossbred animals when compared to female inbred animals, although this difference was not significantly different. When inbred rat strains differing at the RT1 locus were compared for disease expression, only WAG strain rats (RT1^u) were found to be susceptible to disease induction (Table 3.4).

An interesting feature of crossbred Tx-X animals was the high variability in disease incidence levels between groups, particularly for female animals. The disease-onset profiles of three separate groups of crossbred animals established over a period of approximately 12 months are shown in Figure 3.11. The first group established showed an increased susceptibility to disease induction in males when compared to females (60 and 40 percent respectively) with the majority of animals becoming diabetic before 40 days post irradiation (Figure 3.11, group1). Subsequent groups followed the same pattern for males, but incidence rates in females tended to vary,
Table 3.4. Incidence of diabetes and islet lesions in Tx-X rats of differing genetic background.

<table>
<thead>
<tr>
<th>Strain *</th>
<th>RT1 Haplotype</th>
<th>n</th>
<th>Diabetes</th>
<th>Islet Lesions § §</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Acute †</td>
<td>Chronic $ $</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PVG</td>
<td>c</td>
<td>68</td>
<td>20 (29)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>PVG/WF</td>
<td>c/u</td>
<td>30</td>
<td>15 (50) $ $</td>
<td>0</td>
</tr>
<tr>
<td>WAG</td>
<td>u</td>
<td>6</td>
<td>1 (17)</td>
<td>0</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PVG</td>
<td>c</td>
<td>58</td>
<td>21 (36)</td>
<td>3 (5)</td>
</tr>
<tr>
<td>PVG/WF</td>
<td>c/u</td>
<td>56</td>
<td>21 (38)</td>
<td>5 (9)</td>
</tr>
</tbody>
</table>

* No disease observed in Lou/m (n=5), SHR (n=6), DA (n=5) or F344 (n=4) strain female Tx-X rats maintained over the same period

† Hyperglycaemia with insulin-dependence

§ Hyperglycaemia without insulin-dependence

‖ Islet atrophy or insulitis in both diabetic and non-diabetic rats

¶ P < 0.05 compared with PVG female value

‡ P < 0.05 compared with PVG/WF female value
Figure 3.11. Cumulative incidence of acute diabetes in 3 successive groups of Tx-X PVG/WF crossbred rats following final irradiation.
ranging from 0 to 100 percent in consecutive groups (Figure 3.11, groups 2 and 3).

3.4 DISCUSSION

The data presented in this chapter demonstrates that a non-obese, insulin-dependent diabetic syndrome could be induced in rats not normally prone to the spontaneous development of this disease by a combination of early-age thymectomy and low-dose gamma irradiation (Tx-X), a procedure that modulates immune function. The syndrome was characterized by a rapid onset of symptoms including hyperglycaemia, ketoacidosis, insulinopaenia (Table 3.1) and diabetic animals were observed to suffer from polyphagia, polydipsia and polyuria. These observations, in conjunction with the fact that diabetic animals were dependent on exogenous insulin therapy for survival, illustrate that the clinical features of Tx-X - induced IDDM are analogous to those of human insulin-dependent diabetes mellitus.

Onset of diabetes in Tx-X rats was rapid and accompanied by a precipitous weight loss and a concurrent rise in blood glucose levels (Figures 3.2 and 3.3). Diabetic rats became intolerant to exogenous glucose and were dependent on early insulin treatment for survival (Figures 3.3 and 3.4). These features are remarkably similar to those of the diabetic syndromes of both humans and BB rats, but differ from the NOD mouse where disease onset is characterized by less severe clinical features and less dependence on early insulin therapy.

Tx-X induced IDDM had a rapid disease progression, with the majority of diabetic animals developing disease three to four weeks (with some one
or two weeks) after final irradiation (Figure 3.9). The observation that the number of animals developing diabetes reached a peak at four weeks post irradiation suggested that there was a 'window' period of disease susceptibility in Tx-X animals, after which time a relative resistance to diabetes onset developed. Whether this resistance to disease arose as a consequence of restored immunoregulation is not clear, however previous studies have shown that reconstitution of Tx-X rats with lymphoid cells taken from "post-window" Tx-X donors does not restore regulatory capacity, suggesting that this is not the case (Ansar Ahmed et al., 1985).

In contrast to the apparent rapid onset of Tx-X induced IDDM, the diabetic syndromes of both human and spontaneous animal models appear to have as a common feature an extended prediabetic period during which signs of autoimmune activity can be detected. In the Tx-X context, this infers that either an extended pre-disease phase was initiated early in the irradiation schedule or that the disease process in Tx-X PVG rats was more rapid and of a more severe nature, being initiated soon after final irradiation. In support of the latter hypothesis was the observation that autoantibodies to thyroid antigens in Tx-X rats are not detectable until after the final irradiation.

Another feature common to the Tx-X-induced, human and BB rat IDDM syndromes is the close to equal incidence levels of disease in males and females. In Tx-X rats, the incidence of acute disease was slightly elevated in males (36 percent in males compared with 29 percent in females), but this was not statistically significant (Table 3.3). The frequency of islet lesions, however, was significantly raised in males ($P < 0.05$). Overall, the frequency of acute diabetes in females was 34 percent for all animals examined during the course of this study compared
to 36 percent in males, suggesting that the influence of gender on IDDM inducement is minimal in Tx-X rats. In addition, comparison of the disease-onset profiles of male and female rats indicated a similar timing for disease initiation and a similar rate of disease onset (Figure 3.10). This is in contrast to the NOD mouse, where onset of disease is earlier and incidence levels are higher in females (see section 2.3.1(ii)).

Interestingly, for thyroiditis induction by Tx-X, females are markedly more susceptible, and in this case strong evidence has been presented indicating that steroid hormones can influence thyroiditis expression (Ansar Ahmed and Penhale, 1982; Ansar Ahmed et al., 1983).

The influence of genetic differences at the MHC on the level of Tx-X induced IDDM was also investigated. Only WAG inbred rats of RT1<sup>u</sup> haplotype were found to be susceptible to Tx-X induced IDDM when compared to other rat strains, but only with a low frequency (Table 3.4). Interestingly, the RT1<sup>u</sup> haplotype has been found to be essential for IDDM development in BB rats (see section 2.4.1). When this haplotype was introduced onto the PVG genetic background by crossbreeding with an RT1<sup>u</sup> (WF) strain, however, a highly variable pattern of incidence levels was observed between individual groups of animals and also between male and female animals (Figure 3.11). The majority of this variability was confined to female RT1<sup>c/u</sup> hybrid animals, in which disease incidence ranged from 0 to 100 percent in sequential groups compared to a less dramatic range of 40 to 60 percent in males. This high degree of variability must have been largely genetically determined, and suggests that the WF strain used for crossbreeding may not have been fully inbred and thus not homozygous at the MHC for RT1<sup>u</sup>. These observations therefore indicate that the RT1<sup>u</sup> haplotype is not essential for diabetes
induction by the Tx-X procedure, but has the potential to confer enhanced disease susceptibility when introduced onto the PVG background.

The ability of RT1\textsuperscript{u} to enhance the susceptibility of PVG rats to Tx-X induced diabetes has been clearly demonstrated by the recent studies of Fowell and Mason, who utilized the Tx-X protocol to induce IDDM in PVG.RT1\textsuperscript{u} congenic rats (Fowell et al., 1991; Fowell and Mason, 1993). One hundred percent of male rats and 70 percent of females developed disease, suggesting that introduction of RT1\textsuperscript{u} onto the PVG genetic background does act to enhance Tx-X-induced diabetes expression. Together, this data clearly indicates a multigenic influence on disease susceptibility involving genes both within and outside the MHC.

The most common histopathological lesion of diabetic Tx-X rats was islet atrophy, where islets showed a reduction in cell number and size, while focal atrophy or insulitis were more common in less severely affected or clinically normal animals (Figure 3.5 and Table 3.2). Immunofluorescent staining of the various islet lesion types demonstrated that the loss of cell numbers in atrophic islets was due to a specific destruction of $\beta$ cells, and therefore characteristic of the end-stage atrophy seen in other IDDM syndromes. $\beta$ cells were still demonstrable in infiltrated islets and other studies have shown that this infiltrate is composed of both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells, macrophages, dendritic cells and NK cells (Fowell et al., 1991). This suggests a dynamic disease process where lymphocytes invade the islet and then, once $\beta$ cell destruction is complete, migrate rapidly from the lesion site, which manifests clinically as an acute syndrome. In addition however, the observations that 1) a "chronic" disease process was observed in some animals, where islet lesions and blood glucose changes could be detected but animals did not become
insulin-dependent, and 2) a large proportion of clinically normal animals had insulitis at necropsy, suggest that an additional trigger is required to make the transition from the initiation of islet attack to the complete destruction of $\beta$ cells and development of the insulin-dependent syndrome.

The association of Tx-X induced IDDM with lymphocytic thyroiditis provides further evidence that the diabetes was immune mediated and not the result of irradiation-induced damage per se (Figure 3.7). Thyroiditis is also a common feature of other animal models of IDDM, however the observations that 1) the peak onset time of the two conditions did not coincide; 2) that thyroiditis and IDDM were not always associated in the individual animal and 3) incidence levels of diabetes and thyroiditis differed, suggested that separate and specific initiating signals are involved in the pathogenesis of the two syndromes.

An interesting feature of Tx-X induced IDDM was the high variability in incidence levels of disease seen between successive groups of genetically identical inbred animals maintained under similar conditions, and the fact that these incidence levels never reached 100 percent (Figure 3.5). This implies that non-genetically determined factor(s) influence the onset of disease in these animals. As none of the common viral pathogens could be detected (see section 3.2.7) it was hypothesized that flora of the gastrointestinal tract could be the source of this environmentally-determined factor. This inference was given support by previous studies demonstrating that the level of thyroiditis in SPF rats could be increased by the oral administration of fresh gastrointestinal contents from conventionally-derived rats (Penhale and Young, 1988). To test this hypothesis, the ability to induce disease in genetically identical
PVG rats derived under either clean (SPF) or non-barrier maintained conditions was compared. Surprisingly, conventionally-derived rats showed a striking resistance to disease induction compared with rats of SPF origin. Since these rats were of identical genotype, this suggests that in this case an environmentally-derived (gastrointestinal?) factor was encouraging a protective (suppressive?) immune response. It would thus be of interest to examine the cytokine profiles of T cells from these protected animals for enhanced production of so-called 'suppressive' cytokines. Administration of IL-4, for example, has been shown to prevent the onset of diabetes in NOD mice (Rapoport et al., 1993), while certain infectious agents have been shown to induce an IL-4 response by CD4+ T cells (Rocken et al., 1992).

In a previous study, administration of intestinal contents from conventional rats to SPF animals markedly increased the susceptibility of these "conventionalized" SPF animals to thyroiditis inducement by Tx-X (Penhale and Young, 1988). In addition, these workers also observed a 100 percent incidence of thyroiditis in conventional female PVG Tx-X animals, a level not observed in the present study. This data suggests that in the case of thyroiditis induced by this procedure, the environment can act to trigger disease onset, perhaps through the increased availability of cross-reactive autoantigenic epitopes.

Taken together these results suggest that environmental factors can act either to enhance or suppress the onset of autoimmunity depending on the nature of the disease and/or the environmental factor. In addition, that the incidence of thyroiditis can be enhanced by increased environmental exposure, while diabetes onset is suppressed, suggests that different triggering mechanisms may be operating for the two diseases. This
dichotomy of environmental influence on the course of autoimmune
disease induction within the same experimental system provides a unique
opportunity to analyze the complex role of environmental factors in the
pathogenesis of autoimmune disease.

In conclusion, the diabetic syndrome observed in Tx-X rats is similar to
human IDDM both clinically and pathologically. This syndrome also
closely parallels that of the spontaneous syndromes observed in the BB
rat and NOD mouse, although differs from the latter in some respects.
Disease induction by the Tx-X procedure is strain related and can be
modified by, but is not dependent upon, the presence of the diabetes-
determining RT1u MHC haplotype of BB rats. Furthermore, disease
susceptibility is also highly influenced by the environmental background of
the animal, implicating a strong role for environmentally-derived factors in
the pathogenesis of the disease. Finally, the suitability of this model for
genetic studies, and the ability to manipulate it either towards disease
induction or inhibition should provide further scope for detailed study of
the many interacting factors influencing the pathogenesis of diabetes.
CHAPTER 4.0
HUMORAL IMMUNITY STUDIES
CONTENTS

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4.1 INTRODUCTION

The disruption of immunological tolerance to self components that occurs in autoimmune disease is characterized by the generation of both antibody and cellular immunoreactivity towards self antigens. In organ-specific autoimmune disease, an early sign of autoreactivity is often the generation of autoantibodies directed towards components of the target organ, such as ICA in human IDDM (see section 2.2.4 (i)). In addition, autoantibodies can be detected to target tissues in the early stages of other organ-specific autoimmune diseases such as the thyroid in thyroiditis, gastric parietal cells in autoimmune gastritis and liver mitochondrial components in primary biliary cirrhosis (Roitt et al., 1964; Masala et al., 1980; Frazer et al., 1985).

The role of antibody in the pathogenesis of autoimmune disease, however, has been difficult to establish primarily as a result of the inability to define target autoantigens. While it is possible that autoantibodies themselves may be pathogenic, it is more likely that their presence indicates a disruption of immunological tolerance to a tissue component to which a cellular immune response is generated. The detection of autoantibodies and the identification of the target autoantigens concerned is thus essential for the identification of potential cellular targets and for the development of specific prophylaxis or therapy for these diseases.

Recently, examination of autoantibody specificities generated in a variety of autoimmune conditions has identified a number of putative autoantigens. These include thyroglobulin and thyroid peroxidase in autoimmune thyroiditis, the acetyl choline receptor in myasthenia gravis and myelin basic protein in multiple sclerosis. In addition, a number of
these molecules have been identified as enzymes involved in cellular processes of the target organ, for example thyroid peroxidase (an enzyme involved in thyroglobulin processing) in autoimmune thyroid disease, the H⁺K⁺ ATPase (gastric proton pump) in autoimmune gastritis, the pyruvate dehydrogenase complex in primary biliary cirrhosis and glutamic acid decarboxylase (GAD) in IDDM (Banga and McGregor, 1991). Although shown in vitro to inhibit enzyme activity and also in vivo in some cases, these autoantibodies generally cannot transfer disease to unaffected recipients and their role in the pathogenesis of autoimmune disease has not yet been established.

Several approaches are available for the detection and analysis of antibody specificities in autoimmune disease. The classical approach has been to analyze antibody binding to whole tissue sections of the target organ, usually visualized by immunofluorescent staining. A good example of this approach was the detection of autoantibodies to islet cells (ICA) in IDDM as described in section 2.2.4(i) and other examples include the detection of anti-microsomal antibodies in autoimmune thyroid diseases (Khoury et al., 1984; Banga et al., 1986), primary biliary cirrhosis (Frazer et al., 1985) and other autoimmune disorders (Betterle et al., 1985), and the demonstration of parietal cell antibodies in autoimmune gastritis (Masala et al., 1980). Immunofluorescence allows the visual localization of antibody binding within the tissue, but suffers from a lack of sensitivity and reproducibility and has limited use as a quantitative assay.

The enzyme-linked immunosorbent assay (ELISA) is a variation on the immunofluorescent approach that utilizes an enzyme-linked secondary antiserum in conjunction with a coloured enzyme substrate to detect antibody bound to antigen immobilized on a solid matrix. The ELISA
system is widely used as a quantitative assay and has been used to determine antibody levels to mitochondrial (Surh et al., 1988; Zurgil et al., 1989), gastric parietal cell (Chuang et al., 1992) and thyroid autoantigens (Chan et al., 1987; Kohnno et al., 1988) and pancreatic components (Moncayo et al., 1988). This technique is more sensitive than immunofluorescence and is quantitative, but requires purified antigens in order to determine sub-cellular antibody specificity.

Although the above described assays provide information on the tissue or cellular specificity of autoantibody binding, they do not allow sub-cellular identification of multiple autoantigens. Immunoblotting is a technique that utilizes the separation of intracellular components by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) combined with the electro-transfer of the denatured proteins to a solid-phase for subsequent detection by antibody (Laemmli, 1970; Towbin et al., 1979). This technique has allowed the analysis of antibody specificity at the sub-cellular level in a number of autoimmune diseases including thyroiditis (Banga et al., 1984; Hamada et al., 1987), primary biliary cirrhosis (Fusconi et al., 1992) and to a limited extent in IDDM (Karounos et al., 1990).

While immunoblotting has had many useful applications in the study of antibody specificity generated in autoimmune diseases, the denaturation of proteins before reaction with antibody often produces a heterogeneous pattern of antibody specificities that can be difficult to interpret. This was well illustrated by the inconclusive immunoblotting results seen with IDDM sera when islet cell extracts were used as antigen (Karounos et al., 1990; Karounos and Thomas, 1990; Tuck et al., 1992). In addition, antibodies
that recognize conformational epitopes on a protein antigen will not be
detected under the denaturing conditions of this technique.

Immunoprecipitation is an alternative to immunoblotting that maintains the
integrity of the native antigen and allows detection of antibodies that
recognize conformational epitopes. In this procedure, intracellular
proteins are metabolically labelled with a radioactive isotope, then
immunoprecipitated from the cellular extract by specific antibody,
harvested and analysed by SDS-PAGE and autoradiography. A good
example of the value of this technique was described in section 2.2.4(i) for
the identification of the 64kDa islet cell protein in IDDM, and the technique
has also been used to analyze antibody specificities to autoantigens in
other autoimmune diseases such as thyroid microsomal antigens in
thyroiditis (Kajita et al., 1985; Maastricht et al., 1992).

This chapter describes the use of the above techniques to analyze
autoantibody specificities to islet and other tissues generated by the Tx-X
procedure. The relationship of these autoantibody specificities to those
generated in human IDDM and other animal models of diabetes is
discussed.
4.2 MATERIALS AND METHODS

4.2.1 Immunofluorescent Assays

i) Detection of Islet Cell Antibodies

Islet cell antibodies (ICA) were initially detected by immunofluorescent assays performed on sections of protease treated, formalin-fixed canine tissue. Later assays were performed on cryostat-sections of human Type O pancreatic material in conjunction with the Department of Internal Medicine, University Hospital, Uppsala, Sweden. Tissue sections were overlayed with diabetic or normal rat serum diluted 1:50 in phosphate buffered saline (PBS - see section 5.2.1) containing 1 percent aprotinin (Boehringer Mannheim GmbH, Mannheim, Germany) and 10mM benzamidine-HCl (Sigma Chemicals, Mo, USA) and incubated overnight at 4°C. The sections were then washed 3 times in PBS and overlayed with a 1:40 dilution of anti-rat IgG-FITC (Dakopatts, Denmark). After 2 hours at room temperature the sections were washed, mounted and viewed under a fluorescent microscope (Olympus, Japan).

ii) Detection of Thyroid Autoantibodies

Autoantibodies to thyroid components were detected by immunofluorescence in a similar manner to ICA using protease-digested, formalin-fixed sections of normal rat thyroid. Tx-X rat serum samples were diluted 1:50 in PBS and incubated on tissue sections overnight and then overlayed with anti-rat IgG-FITC (Silenus, Melbourne, Australia) as described above.
4.2.2 Enzyme-linked Immunosorbent Assay (ELISA)

i) Preparation of Antigens

Insulin
To detect autoantibodies to insulin (IAA), a commercial preparation of porcine insulin (CSL Novo, Melbourne, Australia) was diluted to 1µg/ml in ELISA coating buffer (15mM Na₂CO₃, 35mM NaHCO₃, pH 9.6).

Rat Pancreas Extract
Pancreatic autoantibodies (PAA) were detected according to the method of Moncayo et al., 1988. Briefly, adult normal rat pancreata were minced in ice-cold 20mM Tris-HCl (pH 7.4), homogenized in a glass homogenizer and filtered through one-ply of gauze. The filtrate was then centrifuged at 8000xg for 30min at 4°C (Sorvall®, DuPont, USA) and then ultracentrifuged at 80000xg for 30min (Beckman, USA). Protein concentration was determined by the Bradford assay (Bio-Rad, CA, USA) and preparations were adjusted to 5µg / ml in ELISA coating buffer.

Rat Thyroglobulin
Four grams of rat thyroids were placed in 10 ml of ice-cold borate buffer (12.5mM Na₂B₄O₇, 150mM NaCl, pH 7.4) and homogenized in a Polytron PCU-2 tissue homogenizer (Kinematica, Luzern, Switzerland) on ice until all tissue had been disrupted. After standing on ice for 30 minutes, the thyroid extract (TgE) was filtered through cotton wool, centrifuged at 21000xg for 30 minutes at 4°C and the supernatant stored at -80°C. To prepare purified rat thyroglobulin (rTg), 1 to 2ml of TgE was loaded onto a K26/100 sephacryl S-300 molecular-weight exclusion chromatography column (Pharmacia, Uppsala, Sweden) and run overnight at 4°C.
Fractions representing the major peak were collected, concentrated in a Centriprep® microconcentrator (Amicon, MA, USA) and adjusted to 25µg/ml in ELISA coating buffer.

ii) ELISA Protocol

Antigen preparations diluted in coating buffer were used to sensitize 96-well microtitre plates (Disposable Products, Adelaide, Australia). One hundred µl of each antigen solution was added per well and incubated at 4°C for 24 hours in the case of whole pancreas and purified rTg, or for 48 hours in the case of the porcine insulin preparation.

After sensitization, plates were washed 3 times with an 0.05 percent solution of Tween-20 (Boehringer Mannheim GmbH, Mannheim, Germany) in PBS (PBST) and non-specific binding sites blocked using PBS containing 0.5 percent Tween-20 for 1 hour at room temperature. One hundred µl per well of rat serum diluted 1:50 in PBST was then incubated overnight at 4°C, followed by an anti-rat IgG-alkaline phosphatase conjugated antiserum (Bio-Rad, CA, USA) diluted 1:5000 in PBST and incubated for 2 hours at room temperature. Bound antibodies were detected colorimetrically using a 1mg/ml solution of the alkaline phosphatase substrate 4-nitrophenol phosphate (4-NPP; Boehringer Mannheim GmbH, Mannheim, Germany) diluted in substrate buffer (10 percent v/v diethanolamine, 0.05mM MgCl₂, pH9.8). After 15 to 30 minutes at room temperature optical density was determined at 405nm (Titertek Multiskan®; Flow Laboratories, Helsinki, Finland).
4.2.3 Immunoprecipitation Assay

i) Preparation of Rat Islets

Pancreatic islets were isolated from 200 to 250 gram male PVG rats by a modification of the collagenase digestion technique (Wollheim et al., 1990). Animals were killed, bled by cardiac puncture and the common bile duct exposed and clamped at the entry point to the duodenum. The duct was then cannulated toward the hilus of the liver and 5ml of a 1mg/ml solution of collagenase Type V (Sigma Chemicals, MO, USA) dissolved in Hank's Balanced Salt Solution containing HEPES (HBSS-HEPES - see section 5.2.1) was injected slowly until the pancreas was fully distended. The tissue was then removed to a siliconized and sterile container, incubated for 12 minutes at 37°C, resuspended in ice-cold HBSS-HEPES and disrupted by vigorous shaking for 10 seconds. After washing 2 times in ice-cold HBSS-HEPES containing 0.35 percent bovine serum albumin (HBSS-HEPES/BSA) by spinning briefly at 2000 rpm and vigorously resuspending through a constricted pipette tip, tissue pieces were filtered through nylon mesh (pore size approximately 0.5mm$^2$) and washed again in HBSS-HEPES / BSA. The final washed pellet was then resuspended to a volume of 10ml per pancreas in Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden), carefully overlayed with an equal volume of HBSS-HEPES/BSA warmed to room temperature and centrifuged at 900xg for 20 minutes at 18°C. Purified islets were collected from the interface, washed a further 3 times in HBSS-HEPES/BSA using a sterile, siliconized centrifuge tube and then resuspended in complete RPMI (see section 5.2.1). The islets were then hand-picked twice under a stereo-microscope.
ii) \(^{35}\text{S}\)-methionine Labelling of Islets and Immunoprecipitation of Islet Cell Proteins

Purified islets were resuspended to 1000 in 2ml of complete RPMI and allowed to recover overnight at 37°C in 5 percent CO\(_2\). The islets were then washed once in methionine-free RPMI (Gibco BRL, MD, USA) then resuspended to 500 islets in 2ml of labelling medium (methionine-free RPMI containing 20mM HEPES, 0.2 percent BSA, 16.7mM D-glucose, 2mM glutamine, 0.04 percent NaHCO\(_3\) and 0.05mM 2-ME, pH7.4) and incubated for 30 minutes prior to adding 0.5 mCi of \(^{35}\text{S}\)-methionine (Amersham Int., Amersham, UK) per 500 islets. After incubating for 7 hours at 37°C in 5 percent CO\(_2\), labelled islets were then washed twice in HBSS-HEPES containing 0.2 percent BSA and 0.01 percent D-L methionine and once in 150mM NaCl, 10mM HEPES, 0.01 percent D-L methionine, 10mM benzamidine-HCl (pH 7.4), then resuspended to 250 islets per 100µl of ice-cold extraction buffer (150mM NaCl, 20mM Tris-HCl pH7.4), 2 percent v/v Triton X-100 [Boehringer Mannheim GmbH, Mannheim, Germany], 0.5 percent aprotinin, 10mM benzamidine HCl, 0.1mM \(p\)-chloromercuribenzenesulphonic acid). The islets were then disrupted on ice using a glass homogenizer, extracted for a further 2 hours on ice and centrifuged at 10 000xg for 30 minutes at 4°C in a refrigerated microfuge (International Equipment Company, MA, USA). The supernatant was collected, divided into 100µl aliquots and stored at -80°C.

To immunoprecipitate islet cell proteins, aliquots of labelled islet extract were thawed on ice and pre-cleared by adding 25µl of normal rat or human serum and incubating overnight at 4°C. Immune complexes were then removed on protein-G Sepharose (PGS (Pharmacia, Uppsala, Sweden) by incubating for 1 hour at 4°C and then microcentrifuging for 30
seconds at maximum speed (Clements, Sydney, Australia). Twenty five μl of test rat or human serum was added to the supernatant and incubated overnight at 4°C. Immune complexes were recovered on PGS for 2 hours at 4°C followed by micro-centrifugation, and the PGS pellet washed in 6 changes of 150mM NaCl, 20mM Tris-HCl (pH7.4), 0.05mM 2-ME, 1 percent v/v Triton X-100).

iii) SDS-PAGE and Autoradiography

Immunoprecipitated proteins were visualized by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography. Proteins bound to the final washed PGS pellet were solubilized in sample buffer (0.5mM Tris-HCl [pH6.8], 2 percent SDS, 10 percent glycerol, 5 percent 2-ME, 0.001 percent bromophenol blue) by resuspending the pellet in 50μl and boiling for 3 minutes. The samples were then quickly centrifuged and loaded onto 16cm x 15cm x 0.75mm SDS-polyacrylamide slab gels (Protean II electrophoresis system - Bio-Rad, CA, USA) consisting of a 10 percent acrylamide resolving gel in 1.5mM Tris-HCl (pH 8.8) and a 4 percent acrylamide stacking gel in 0.5mM Tris-HCl (pH 6.8). Electrophoresis was at 40mA constant current for 3.5 hours using the buffer system of Laemmli (Laemmli, 1970). Commercial molecular weight standards were run on each gel (Sigma Chemicals, MO, USA). Following electrophoresis, gels were stained for 1 hour in 0.2 percent Coomassie Blue (Koch-Light, Suffolk, UK) and fixed overnight in destainer (2 percent v/v glacial acetic acid, 30 percent v/v methanol).

Destained gels were processed for fluorography by soaking in Amplify (Amersham Int., Amersham, UK) for 30 minutes followed by drying at
70°C for 3 hours in a slab-gel dryer (Bio-Rad, CA, USA). The dried gel was then loaded into an autoradiography cassette with intensifying screens (Amersham Int., Amersham, UK) and exposed to Kodak X-Omat AR X-ray film at -80°C for 2 to 4 weeks.

4.2.4 Immunoblotting

To determine the binding specificities of anti-islet cell antibodies, islet cell proteins were subjected to SDS-PAGE and immunoblotting. SDS-PAGE conditions were as described in 4.2.3. Purified rat islets were resuspended to a concentration of 2x10^3 / ml in sample buffer and boiled for 5 minutes. Islet extract was then loaded onto preparative SDS-PAGE gels consisting of a 15 percent resolving gel and 4 percent stacking gel. 600µl of sample was loaded for 16cm x 15cm x 0.75mm gels, and 200µl for 10cm x 6cm x 0.5mm gels (mini-Protean II system - Bio-Rad, CA, USA). Molecular weight standards (Sigma Chemicals, MO, USA) were also included.

Following electrophoresis, separated proteins were transferred to nitrocellulose membrane (Hybond C - Amersham Int., Amersham, UK) using the Transblot or mini-Transblot transfer systems (Bio-Rad, CA, USA) and transfer buffer (25mM Tris-HCl, 192mM glycine, 20 percent v/v methanol, pH 8.3). Transfer chambers were cooled to 4°C and transfer conditions were 100 volts constant for 1 hour using the mini-Transblot system or 30 volts constant overnight for the Transblot system. After transfer, nitrocellulose membranes were either cut into strips or left intact, blocked in 3 percent BSA in PBS for 30 minutes at room temperature and washed three times in PBST. In some cases nitrocellulose membranes were stained for total protein using amido black stain (Bio-Rad, CA, USA).
For large-scale screening of serum samples, intact nitrocellulose membranes were loaded on a Miniblotter® screening chamber (Immunetics, MA, USA) and test or control rat sera diluted 1:50 in PBST loaded into each lane. In some cases individual nitrocellulose strips were incubated with larger volumes (2ml) of diluted sera. Membranes were incubated overnight at 4°C, washed and then incubated with an anti-rat IgG - horseradish peroxidase conjugated antiserum (BioRad, CA, USA) diluted 1:3000 for 1 hour at room temperature. Bound antibodies were then detected by the Enhanced Chemiluminescent (ECL) system (Amersham Int, Amersham, UK) using Fuji RXO-G X-ray film and 30 second to 2 minute exposures.

4.3 RESULTS

4.3.1 Detection of Autoantibodies by Immunofluorescence

i) Islet Cell Antibodies (ICA)

Typical immunofluorescent staining patterns for diabetic sera on islet sections are shown in Figure 4.1. Initially, to avoid high background staining seen with rat pancreas, canine pancreas sections were used as substrate. ICA were detected under these conditions in 4/16 (25 percent) of plasma samples taken from acute diabetic rats (Figure 4.1a). These antibodies had a range of titres from 2 to 16, indicating that they were present in low concentrations. Antibody staining was seen across the whole islet, showing no specificity for β cells (whole-islet staining). ICA were not detected in clinically unaffected Tx-X, or normal PVG rats (n=23).
Figure 4.1. Immunofluorescent staining of islets. Formalin-fixed canine or frozen human islet sections were incubated with sera from diabetic rats taken at time of disease onset, then overlayed with an anti-rat IgG-FITC conjugate and photographed under a fluorescent microscope.

a) Canine islet (x400).

b) Human islet (x250).
ICA were also detected with low frequency in diabetic sera using human islet tissue as substrate. For the 100 serum samples screened, 8 were consistently positive, and all gave a 'granular' staining pattern that appeared to be $\beta$ cell selective (Figure 4.1b). ICA were not detected under these conditions in clinically unaffected Tx-X or normal PVG rats (n=20).

ii) Thyroid Autoantibodies

Autoantibodies to thyroid components were detected by immunofluorescent staining of rat thyroid sections in a significant proportion of Tx-X rats (Figure 4.2). Strong reactivity was detected towards both thyroglobulin and microsomal components. This type of antibody was detected in 100 percent of serum samples from rats showing thyroid lesions at necropsy (n=36) and 64 percent of samples from Tx-X rats with normal thyroids at necropsy (n=34). In general, the intensity of fluorescence correlated with the severity of the thyroid lesion.

4.3.2 Detection of Autoantibodies by ELISA

i) Pancreatic Autoantibodies

In an attempt to develop an assay system for the rapid detection of autoantibodies to pancreatic components, whole pancreas extract was used as antigen in an ELISA system. Autoantibodies to pancreas extract were detected at levels significantly greater (optical density of >2 S.D.) than those of normal animals in 3 of 3 acute diabetic plasma samples taken at onset of disease and in 8/27 (30 percent) of non-diabetic terminal plasma samples representing animals from a single Tx-X group.
Figure 4.2. Immunofluorescent staining of rat thyroid section with serum from a thyroiditic Tx-X rat showing antibody binding to microsomal components (x 250).
3/8 (38 percent) of animals that were positive had pancreatic lesions at necropsy as compared to 4/19 (21 percent) of animals that were negative for this type of antibody (Table 4.1).

ii) Insulin Autoantibodies (IAA)

IAA were detected in 5/26 (19 percent) of acute diabetic animals and in 5/29 (17 percent) of clinically non-diabetic Tx-X rats that developed islet lesions (Table 4.1). The incidence of IAA was significantly raised when compared with Tx-X rats with normal islet histology (0/36; \( P < 0.001 \)).

iii) Thyroglobulin Autoantibodies (TgAA)

TgAA were detected in a high proportion of both acute diabetic and clinically unaffected Tx-X rats (Table 4.1). 30 percent (12/40) of acute diabetic animals and 48/107 (45 percent) of non-acute diabetic Tx-X animals examined during this study had significantly raised TgAA levels. Of the latter group, TgAA were detected in 8/24 (33 percent) of Tx-X rats with islet lesions at necropsy and 40/83 (48 percent) of Tx-X rats with normal pancreas histology. Generally, raised TgAA levels correlated well with the presence of thyroid lesions in the individual animal, with 7/12 (58 percent) of TgAA positive acute diabetic animals and 41/48 (85 percent) of TgAA positive non-diabetic Tx-X animals showing signs of mild to severe thyroid infiltration.
Table 4.1. Incidence of autoantibodies to pancreas extract, thyroglobulin and insulin in Tx-X rats as determined by ELISA *

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Acute Diabetic</th>
<th>Tx-X / Non clinically-diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>With Islet Lesions</td>
</tr>
<tr>
<td>Pancreas</td>
<td>3/3 (100%)</td>
<td>3/8 (38%)</td>
</tr>
<tr>
<td>Extract</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>5/26 (19%)</td>
<td>5/29 (17%)</td>
</tr>
<tr>
<td>rTg</td>
<td>12/40 (30%)</td>
<td>8/24 (33%)</td>
</tr>
</tbody>
</table>

* Positive value calculated as an O.D.405 reading of 2 SD or greater than mean value for normal PVG controls (n > 10).
† P<0.001 compared with Tx-X rats with islet lesions and acute diabetic rats.
4.3.3 Immunoblotting Assays

Using the high sensitivity of the chemiluminescent reaction, immunoblotting of diabetic sera against islet proteins revealed antibody specificities to a number of protein bands. Initial experiments revealed reasonably strong antibody reactivity in diabetic sera reacting to islet proteins with a range of molecular weights (Figure 4.3 - lanes 1 to 12), with some weaker reactivity observed in normal sera (Figure 4.3 - lanes 13 to 16). Protein bands recognized by diabetic sera ranged in molecular weight from 19 to 52kDa, with the strongest reacting bands at 19kDa (Figure 4.3 - lane 1) and 33kDa (Figure 4.3 - lanes 4 and 10). It was not possible, however, to identify antibody reactivity to a single disease-associated protein band in diabetic sera.

A similar pattern was seen when a larger group of diabetic and normal sera were screened on a different occasion using a different islet extract (Figure 4.4). Although in this experiment a greater degree of reactivity in normal serum was observed (lanes 21 to 36), this was generally weaker and directed towards bands other than those recognized by diabetic sera (lanes 1 to 20). Strong reactions were again observed to the 33kDa band in a number of sera (lanes 1, 5, 10, 17, 19) and also to some higher molecular weight bands of 76 and 114kDa (lanes 10 and 12 respectively). Again however, no single disease-associated band could be identified.

Serial dilution of diabetic sera showed that antibody titres to these bands ranged from 100 for the 33kDa band, to approximately 400 for the 19kDa band and 1600 for the 52kDa band (Figure 4.5).
Figure 4.3. Immunoblotting of diabetic and normal rat sera against islet-cell extract. Rat islet extract was resolved by SDS-PAGE, transferred to nitrocellulose membrane then incubated with acute diabetic (1-12) or normal PVG (13-16) rat sera. Bound antibodies were detected by an anti-rat IgG-peroxidase conjugate and enhanced chemiluminescence.
Figure 4.4. Immunoblotting of acute diabetic (lanes 1-20) and normal (lanes 21-36) rat sera against rat islet cell extract. Method as per Fig. 4.3.

Figure 4.5. Antibody titres of sera from diabetic rats reactive with islet cell extract by immunoblotting. Serum samples previously identified as reacting with different rat islet cell proteins by immunoblotting were serially diluted and re-examined by immunoblotting against rat islet extract.
In addition, serum samples from non-acute diabetic Tx-X rats were also screened for islet reactivity. Rats were divided into two groups on the basis of the presence or absence of islet lesions at necropsy, and screened by immunoblotting against rat islet extract (Figure 4.6). Although some sera did react strongly in both groups to one or more protein bands, overall reactivity was reduced in these sera and there was no difference between serum samples from rats with islet lesions compared to those with normal islet histology.

The frequency of antibodies to the various islet cell protein bands in diabetic and Tx-X sera is summarized in Table 4.2. The most frequently detected band was at 33kDa, being recognized by 8/32 (25 percent) of acute diabetic serum samples and 2/20 (10 percent) of samples from non-acute diabetic Tx-X rats with islet lesions at necropsy. Overall, positive reactions to at least one islet protein band was observed in 24/32 (75 percent) of diabetic serum samples compared to 6/20 (30 percent) of Tx-X rats with islet lesions and 3/20 (15 percent) of Tx-X rats with no islet lesions.

To examine the tissue specificity of islet-reactive antibodies, immunoblotting was performed using liver, kidney, lymph node, testis, salivary and adrenal gland extracts (Figure 4.7a). Diabetic sera reacting with islet proteins reacted only with liver extract, where weak reactions were observed to the 114 and 76kDa bands, and with lymph node extract, where unique bands at approximately 30 and 28 kDa were observed (Figure 4.7b)

Tissue specificity was also confirmed by adsorption experiments, where islet-reactive sera were adsorbed against nitrocellulose membranes
Figure 4.6. Immunoblotting analysis of non-acute diabetic Tx-X rat sera against islet cell extract. Sera were selected from rats that had developed islet lesions, or from those with a normal pancreas histology, at the end of the induction period without signs of hyperglycaemia.
Table 4.2. Frequency of reactivity to islet cell protein bands in serum samples from Tx-X rats by immunoblotting.

<table>
<thead>
<tr>
<th>Protein Band (kDa)</th>
<th>Acute Diabetic</th>
<th>Tx-X with Islet Lesions</th>
<th>Tx-X without Islet Lesions</th>
<th>Normal PVG (non-Tx-X)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>32</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>114</td>
<td>3 (9)</td>
<td>1 (5)</td>
<td>1 (5)</td>
<td>0</td>
</tr>
<tr>
<td>76</td>
<td>2 (6)</td>
<td>1 (5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>52</td>
<td>4 (12)</td>
<td>1 (5)</td>
<td>1 (5)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>49</td>
<td>3 (9)</td>
<td>0</td>
<td>1 (5)</td>
<td>0</td>
</tr>
<tr>
<td>33</td>
<td>8 (25)</td>
<td>2 (10)</td>
<td>0</td>
<td>4 (20) *</td>
</tr>
<tr>
<td>30</td>
<td>2 (6)</td>
<td>0</td>
<td>0</td>
<td>1 (5)</td>
</tr>
<tr>
<td>19</td>
<td>2 (6)</td>
<td>1 (5)</td>
<td>0</td>
<td>1 (5) *</td>
</tr>
<tr>
<td>Total</td>
<td>24 (75)</td>
<td>6 (30)</td>
<td>3 (15)</td>
<td>7 (35)</td>
</tr>
</tbody>
</table>

* Intensity of reactions significantly weaker than those of diabetic sera
Figure 4.7. Tissue specificity of islet-cell reactive diabetic sera.
a) Amido Black stain of tissue extracts resolved by SDS-PAGE and transferred to nitrocellulose membrane. (Is - islet; LN - lymph node; Li - liver; Ki - kidney; Te - testis; Ad - adrenal gland; Sal - salivary gland)
b) Serum samples previously shown to react with islet cell extract by immunoblotting (1, 2 and 3) were immunoblotted against the above tissue extracts.
c) Islet-cell reactive diabetic sera (1-5) were pre-adsorbed against nitrocellulose-bound extracts of liver, kidney and lymph node, then immunoblotted against rat islet cell extract.

![Figure 4.7](image-url)

### Table 4.7

<table>
<thead>
<tr>
<th>Unadsorbed</th>
<th>Pre-adsorbed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
</tr>
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<td>3</td>
<td>3</td>
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<td>4</td>
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**kDa**

<table>
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</thead>
<tbody>
<tr>
<td>52</td>
</tr>
<tr>
<td>33</td>
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</tbody>
</table>
sensitized with SDS-PAGE resolved liver, kidney or lymph node extracts. Repeated adsorption of these sera against the sensitized membranes did not remove reactivity to a range of islet proteins, indicating the islet specificity of these antibodies (Figure 4.7c).

4.3.4 Immunoprecipitation Assay

Preliminary studies using the immunoprecipitation assay to detect antibody reactivity to native islet cell proteins identified antibodies to a 64kDa protein in acute diabetic rat sera (Figure 4.8). This band co-migrated with a similar-sized protein recognized by both human IDDM and Stiff Man Syndrome (SMS) sera - a rare neurological disorder in which a significant proportion of patients develop IDDM and antibodies to a 64kDa islet cell protein (Baekkeskov et al., 1990). At this stage a small number of normal rat serum samples have been negative for anti-64kDa reactivity and studies are currently in progress to screen a larger number of both acute diabetic and normal samples to determine the incidence of these antibodies and their relationship to the disease process.

4.4 DISCUSSION

The data presented above demonstrates that the production of autoantibodies to self-components is a regular feature of Tx-X rats, providing further evidence for an immune-mediated pathogenesis of autoimmunity in this model. Using the classical approach of immunofluorescence, autoantibodies to islet cell antigens were detected in a proportion of acute diabetic Tx-X animals using fixed canine islets as substrate, however these antibodies were difficult to detect and were only
Figure 4.8. Immunoprecipitation of rat islet-cell proteins. Purified rat islets were metabolically labelled with $^{35}$S-methionine then extracted and incubated with serum samples. Immune complexes were then harvested on Protein-G sepharose, separated by SDS-PAGE and visualized by autoradiography.

Lane 1 - 64kDa positive human IDDM serum.
Lane 2 - SMS serum.
Lanes 3 - 6 - acute diabetic Tx-X rat sera.
present in low titres (Figure 4.1a). The staining pattern was similar to that described by Bottazzo et al. for human ICA (Bottazzo et al., 1974) where the whole islet was stained with no apparent specificity for particular cell types. In contrast, a restricted number of sera from diabetic Tx-X animals demonstrated a "granular", β-cell specific staining pattern when human tissue was used as substrate (Figure 4.1b), similar to that recently described for human ICA (Genovese et al., 1992; Timsit et al., 1992). Again however, these antibodies were difficult to detect and were present in low titres, and to date only a small number of animals have been tested. Nevertheless, this is the first report of this particular type of staining pattern in an animal model of IDDM and further studies are required to determine the value of these antibodies in predicting the onset of IDDM in Tx-X rats.

Assay variability appears to be a common feature in the detection of ICA by immunofluorescence, as illustrated an international workshop on the standardization of ICA where a high degree of variability in precision and limits of ICA detection was seen when 15 standard ICA sera were tested in 41 different laboratories (Bonifacio et al., 1990b). The authors ascribed this variability to a combination of differences in methodology and also variable antigen expression in tissue substrates used. In addition, ICA cannot be detected in another rat model of IDDM, the BB rat (Like et al., 1982a; Elder et al., 1982; Maclaren et al., 1983; Dyrberg et al., 1983). This suggests that a combination of methodology, substrate variability and dealing with low-titre antibodies of rat origin may have contributed to the difficulty in detecting ICA in Tx-X rats by immunofluorescence. This data also questions the significance of the role of these antibodies in the pathogenesis of the disease.
In contrast to ICA, autoantibodies to thyroid antigens were readily detected in Tx-X rats, whether diabetic or otherwise (Figure 4.2). Again, a number of tissue components were stained including thyroglobulin and thyroid follicular cells, illustrating the polyclonal nature of the antibody response to this tissue.

By ELISA, autoantibodies to soluble antigens could also be detected in Tx-X rats (Table 4.1). Autoantibodies to pancreatic extract were readily detected in all Tx-X animals, but did appear to be more prevalent in acute diabetic animals. Nevertheless, these antibodies were not disease specific in that they did not discriminate between Tx-X rats with islet lesions and those with normal pancreas histology at necropsy. In contrast, autoantibodies to insulin (IAA) did appear to discriminate between Tx-X rats with or without islet lesions, being more prevalent in the former group. No difference was observed, however, in the IAA levels of Tx-X rats with islet lesions and those of acute diabetic animals, with IAA being detected in both groups at a low frequency (17 to 19 percent). The low frequency of IAA seen in diabetic animals limits their use as predictive markers for disease onset, and this is consistent with findings in the BB rat where IAA were either not detected or were present at low levels and had limited predictive value (Dean et al., 1987; Markholst et al., 1990; Diaz et al., 1991). Similarly in humans, IAA appear to have a poor predictive value for disease onset (see section 2.2.4).

In contrast, autoantibodies to purified rat thyroglobulin (TgAA) were a common feature of Tx-X rats, detectable with high frequency in most groups. Both acute diabetic and non-diabetic Tx-X rats with islet lesions showed TgAA incidence levels of around 30 percent, and the frequency of detection increased in Tx-X rats without islet lesions at necropsy. This
suggests that Tx-X rats with no on-going islet autoimmunity are able to generate a more severe thyroid autoimmune response, reflected by the reduced frequency of thyroid lesions in diabetic rats (see Table 3.2). Raised TgAA levels correlated well with the presence of thyroid lesions, and again this correlation was greater in Tx-X rats without on-going islet autoimmunity. Antibody titre however did not correlate with severity of thyroid lesions, which is in accordance with earlier studies in this model demonstrating a relationship between TgAA and thyroiditis incidence, but not TgAA levels and thyroiditis severity (Penhale et al., 1973).

In an attempt to analyze the specificities of antibodies to islet cell proteins, immunoblotting was performed on lysates of purified islet cells. Using this technique, a heterogeneous pattern of immunoreactivity to islet cell proteins was observed (Figures 4.3 and 4.4). This immunoreactivity did appear to be disease related however, in that the majority was confined to diabetic sera (75 percent) and to a lesser extent in sera from non-diabetic Tx-X rats with islet lesions at necropsy (30 percent) or with normal islet histology (15 percent) (Table 4.2). A proportion of normal sera, however, also showed anti-islet cell reactivity by this procedure but in most cases this was directed towards different protein bands than those recognized by diabetic sera or were of a much lower intensity and probably reflects the high sensitivity of this assay procedure. These results are in contrast to those of the immunofluorescent assay, where ICA were difficult to detect and present at low frequency. The response was also tissue specific in that no reactivity was seen to a range of non-islet tissues and could not be removed by prior adsorption to membrane-bound tissue extracts.
The heterogeneous pattern of immunoreactivity seen by immunoblotting illustrates one of the disadvantages of using this technique. A range of protein bands in islet cell extract were recognized, with no single protein being recognized with a frequency of more than 25 percent. This suggests that either a highly polyclonal response to islet cell proteins is generated in diabetic Tx-X rats and/or that these antibodies are recognizing peptides generated during the denaturation and reducing conditions of this technique from a smaller number of native proteins molecules. Although the majority of immunoreactivity (25 percent) was to a 33kDa islet cell protein, the strongest reactivity was directed towards a 52kDa protein in 12 percent of cases. Interestingly, antibodies to a protein of the same molecular weight (52 kDa) were identified with a slightly higher frequency and similar titre by Karounos and associates using human and NOD mouse serum by immunoblotting of human islet cell extracts, suggesting that they may be the same protein (Karounos et al., 1990; Karounos and Thomas, 1990). The 52kDa protein was found to be membrane-bound, but was only detected using the insulinoma cell line RINm5 as antigen and not purified islet cells. This fact, in conjunction with the low frequency of autoantibodies to this protein in human, NOD mouse and Tx-X rat suggests that this antigen may not be of pathogenic significance. In contrast, no immunoreactivity to a 33kDa islet cell protein was reported. These workers also observed a diversity of antibody specificities using this technique and were unable to identify a single disease-associated protein band.

Of particular interest is the identification of antibody reactivity towards a 64 kDa protein band in diabetic Tx-X sera by immunoprecipitation (Figure 4.8). A protein of this molecular weight was not detected under the denaturing and reducing conditions of immunoblotting, indicating that
either anti-64kDa antibodies recognize a conformational epitope on the native antigen or that the antigen is particularly sensitive to denaturation and degradation. This highlights the value of using this technique to detect conformation-restricted antibody binding or binding to native antigens. The assay was not suited to screening large numbers of serum samples however, hence the distribution of antibodies to the 64kDa antigen among different groups of Tx-X could not be determined.

To date anti-64kDa antibodies have proved to be the most consistent immunological feature of human, BB rat and NOD mouse syndromes and has potential as a candidate autoantigen in IDDM (see sections 2.2.4(i), 2.3.1(i) and (ii)). Due to the demanding nature of the immunoprecipitation assay however, further work is required to establish the disease specificity of antibodies to this protein in Tx-X rats and their value as predictive markers of disease onset.

In conclusion, the data presented in this chapter demonstrates that Tx-X rats are capable of generating an autoantibody response towards a range of islet cell and thyroid components which can be detected by a number of assay systems.

The classical approach of immunofluorescence readily detected antibodies to thyroid components, but appeared to have limited value in the detection of islet cell antibodies due to the limitations of lack sensitivity and reproducibility. The ability to more readily detect antibodies to thyroglobulin could be related to the size of the molecule and the presence of multiple epitopes compared to islet cell antigens. Nevertheless, ICA were detected in a small number of diabetic Tx-X rats
and showed a binding pattern to islet cells similar to that seen with human serum.

By ELISA, antibodies to soluble antigens (insulin, pancreas, thyroglobulin) were readily detected, particularly in the case of purified rat thyroglobulin and pancreas extract and the immunoprecipitation assay identified antibodies to a 64kDa islet cell protein similar to that recognized by human sera. In contrast, the increased sensitivity of the immunoblotting assay allowed detection of antibodies to islet cell proteins in a large proportion of diabetic sera, but this technique produced a complex pattern of immunoreactivity that was difficult to interpret. The ability to more readily detect antibody reactivity toward thyroid as compared with islet components may indicate a more active role for autoantibody in the pathogenesis of thyroid disease but suggests that humoral immunity probably does not play a primary role in the pathogenesis of Tx-X induced IDDM.

Finally, this data provides further evidence for disrupted immunoregulation in Tx-X rats and supports an autoimmune pathogenesis for Tx-X induced IDDM.
CHAPTER 5.0
CELL-MEDIATED IMMUNITY STUDIES
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5.1 GENERAL INTRODUCTION

Observations in diabetes and other autoimmune syndromes, both in man and experimental animal models, suggest a strong role for cell-mediated immune mechanisms in the pathogenesis of disease. The presence of insulitis in IDDM, and chronic inflammation of target tissues in thyroiditis, rheumatoid arthritis and autoimmune encephalomyelitis provides local evidence for a cellular immune mechanism in the generation of the autoimmune pathology seen in these diseases. This hypothesis is further supported by the ability to inhibit disease-onset in animal models of IDDM by treatment with anti-lymphocyte serum, immunosuppressive agents or neonatal thymectomy (see section 2.4.2(ii)). Moreover, attempts to induce organ specific autoimmunity by passive transfer of autoantibodies have been almost entirely unsuccessful.

Central to the issue of autoimmunity and maintenance of self tolerance has been the question of how potentially self-reactive lymphocytes, arising as a result of random rearrangement of genes coding for receptor elements, are either eliminated or regulated during development under normal circumstances. This first led to concept of clonal deletion, first proposed by Burnet in 1959, whereby potentially autoreactive clones of lymphocytes are deleted during the early stages of development (Burnet, 1959).

While clonal deletion has generally been accepted as playing a role in the initiation of self tolerance, this mechanism cannot account for a number of observations, including the demonstration of autoreactive lymphocytes in the circulation of normal individuals. This has lead to the existence of the phenomenon of peripheral tolerance mechanisms, whereby autoreactive
clones escaping intrathymic clonal deletion are functionally inactivated upon antigen recognition (anergy) or are actively antagonized by suppressive cells in the periphery (Cruse and Lewis, 1992).

While the mechanisms of clonal deletion and anergy have attracted a considerable amount of attention, relatively less is known about the mechanism of suppression, due mainly to the difficulty of cloning and characterizing suppressive T cells. Nevertheless, the concept of suppression cannot be disclaimed as several studies have demonstrated that reconstitution of diabetes-prone animals with spleen cells from normal donors protect recipients from disease in the case of both BB rats and NOD mice (Burstein et al., 1989; Hutchings and Cooke, 1990). In addition, suppressor T-cell lines capable of inhibiting the adoptive transfer of experimental allergic encephalomyelitis from diseased to normal rats have been generated (Ellerman et al., 1988).

For the Tx-X model, studies have shown that transfusions of lymphocytes from normal, unmanipulated rats after completion of the induction protocol can prevent the onset of autoimmunity in recipient Tx-X animals (Penhale et al., 1976; Fowell and Mason, 1993), suggesting the presence of a population of cells in normal animals capable of inhibiting the action of potentially autoreactive T cells in the periphery.

Considered together, the available data suggests that the development of self tolerance is likely to arise as a consequence of a combination of the above described mechanisms - deletion, anergy and suppression. In addition, increasing evidence suggests that the maintenance of self tolerance is a dynamic process involving a carefully regulated balance between auto-reactive and -suppressive elements of the immune system.
Disruption of this balance may lead to the generation of autoreactivity and autoimmune disease.

The following sections describe a series of *in vivo* and *in vitro* cell studies in Tx-X rats designed to detect the presence of autoreactive cells in these animals, and to examine the role of cellular immunity in the induction and regulation of autoimmunity in these animals.
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5.2.1 INTRODUCTION

The most direct evidence for a role for cellular mechanisms in the pathogenesis of autoimmune disease has come from adoptive-cell transfer studies in experimental animal models. For example, lymphoid cells isolated from donor animals in which autoimmune disease had been induced by immunization with myelin basic protein or thyroglobulin were capable of transferring encephalomyelitis or thyroiditis respectively to naive, syngeneic recipients (McMaster and Lerner, 1967; Ben-Nun et al., 1981; Maron et al., 1983; Takenaka et al., 1984; Kotani et al., 1990). For experimental IDDM, several studies have reported the passive transfer of disease using lymphoid cells isolated from affected donors, both for BB rats and NOD mice (Poussier et al., 1983; Koevary et al., 1983; Wicker et al., 1986a; Wicker et al., 1986b). Furthermore, studies in neonatally-thymectomized mice (Sakaguchi et al., 1982) and Tx-X rats (Fowell and Mason, 1993) have shown that a population of T cells present in normal animals are capable of suppressing the onset of disease in these animals.

Together, these studies suggest provide strong evidence that in these model systems the autoimmune pathology is mediated by a population of autoreactive T cells, which in the normal animal are under control of an active T cell-mediated suppressive mechanism. Disruption of this suppressor/inducer balance unmasks the autoreactive potential of these cells and leads to the autoimmune pathology observed.

The following section describes a series of cell reconstitution and transfer studies in Tx-X rats designed to test this suppressor/inducer imbalance hypothesis and examine the role of cell mediated immune mechanisms in the pathogenesis of Tx-X induced autoimmune disease.
5.2.2 MATERIALS AND METHODS

i) Tissue Culture Reagents and Media

Phosphate Buffered Saline (PBS)

PBS (pH 7.3) was prepared from tablets (Oxoid, Basingstoke, U.K.) to give a final concentration of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄ and 1.5 mM KH₂PO₄.

Ca²⁺-free Krebs-Ringer Bicarbonate-HEPES (Ca²⁺-free KRB-HEPES)

Stock solutions of Ca²⁺-free KRB-HEPES were prepared as follows:

Stock solution A: 633 mM NaCl, 23.8 mM KCl, 5.9 mM KH₂PO₄, 5.9 mM MgSO₄.7H₂O.

Stock solution B: 31.2 mM NaHCO₃, 97.6 mM NaCl.

Immediately prior to use, a working solution consisting of 20 ml Solution A, 16 ml Solution B, 1 ml of 1 M HEPES (N-[2-hydroxyethyl]piperazin-N'-[2-ethanesulfonic acid]) - ICN Biomedicals, NSW, Australia) and 58 ml ddH₂O was prepared. The solution was equilibrated in air for 10 minutes and the pH adjusted to 7.4 with 1 M NaOH.

Hanks Balanced Salt Solution-HEPES (HBSS-HEPES)

HBSS was purchased as a 10-times concentrate from Gibco BRL, MD, USA. A working solution of HBSS-HEPES was prepared by supplementing normal strength HBSS with 20 mM HEPES and 4.3 mM NaHCO₃, adjusting the pH to 7.3 with 1 M NaOH and filter sterilizing.
Complete-RPMI

RPMI-1640 medium was purchased from Gibco BRL, MD, USA. Glutamine, penicillin and streptomycin were from Sigma Chemical Co., MO, USA. Complete-RPMI was prepared by supplementing normal-strength RPMI with 20mM HEPES, 2mM glutamine, 0.05mM 2-ME, 100U/ml penicillin, 100ug/ml streptomycin. The solution was adjusted to pH7.3 with 1M NaOH and filter sterilized. Heat-inactivated FCS or NRS were added prior to use as required.

Mitogens

Concanavalin A (ConA), phytohaemagglutinin (PHA) and pokeweed mitogen (PWM) were purchased from Sigma Chemical Co., MO, USA. Stock solutions were made to a concentration of 200 µg/ml in RPMI and used at a working concentration of 5µg/ml for ConA and PHA, or 1µg/ml for PWM.

ii) Preparation of Rat Spleen Cells

Donor rats were killed by CO2 inhalation and their spleens removed aseptically. Single cell suspensions were prepared by gently pressing chopped tissue through a stainless steel mesh (0.5mm2) then washed three times with HBSS-HEPES. Cell viability was assessed by Trypan Blue exclusion and cell numbers determined using a haemocytometer.
iii) Lymphoid Cell Reconstitution of Tx-X rats

For lymphoid-cell reconstitution experiments, freshly-isolated spleen cells from normal PVG donors were adjusted to $1 \times 10^8$ cells in 1ml of PBS and administered IP to Tx-X rats 24 hours after final irradiation. After the usual disease-development period (10 weeks - see Figure 1), reconstituted animals were killed and blood, pancreas and thyroid analyzed as previously described (chapter 3.0).

iv) Adoptive Cell Transfers

Selection of Cell Donors and Recipients

Spleen cell donors for the transfer of diabetes were selected on the basis of recent-onset acute IDDM which had been stabilized by insulin-therapy. Recipients were young (3 or 6 week old) normal female PVG rats, irradiated adult female PVG rats or Tx-X rats that had not become diabetic 8 to 9 weeks after final irradiation and that showed normal glucose tolerance (see below).

Donors for the adoptive transfer of thyroiditis were post-diabetic stage Tx-X rats (8 to 10 weeks after final irradiation) that showed elevated levels of autoantibodies to rat thyroglobulin by ELISA (see section 4.3.2). Recipients were either normal or gamma-irradiated (2.5 Gray 24 hours prior to cell transfer) age-matched female PVG rats.
Preparation and Transfer of Spleen Cells

Single-cell spleen suspensions were prepared as described above, depleted of erythrocytes by hypotonic lysis and resuspended to a concentration of $2 \times 10^6$ cells per ml in complete RPMI supplemented with 10 percent FCS or 5 percent NRS and 2.5µg/ml ConA. Cells were cultured for 72 hours in 5 percent CO$_2$ at 37°C in plastic tissue culture vessels (ICN Biomedicals, NSW, Australia) using 10ml volumes. Cells were prepared for transfer by harvesting and washing 3 times in PBS. Clumps were dispersed by gently passing the solution through a fine-gauge needle and the cells checked for viability and counted. Washed SpC preparations were resuspended to the required concentration in a volume 0.5 ml of PBS and transferred to recipients IV via the caudal vein. Recipients were then killed ten days after cell transfer and blood, pancreas and thyroid analyzed as previously described (chapter 3.0).

Glucose Tolerance Testing

In the case of Tx-X recipients of diabetic cells, intraperitoneal glucose tolerance tests (IPGTT) were performed in order to select appropriate recipients prior to cell transfer, and also following transfer to test for abnormal glucose regulation. Blood glucose levels were determined immediately prior to the IP administration of a solution of D-glucose (BDH Chemicals, Poole, U.K.) in PBS (2mg glucose per gram body weight) and thereafter at 30, 60, 120, and 300 minute intervals. Tests were performed at the same time of day on non-fasted animals and only those showing a blood glucose increase within normal limits were selected as cell-transfer recipients. Recipient animals, together with Tx-X controls, were re-tested...
as above for glucose intolerance 10 days after transfer then necropsied and examined for hyperglycaemia and islet lesions.

5.2.3 RESULTS

i) Lymphocyte-reconstitution of Tx-X Rats

The transfer of viable lymphoid cells isolated from normal (non-Tx-X) PVG donors to Tx-X rats shortly after final irradiation was effective in inhibiting the onset of both diabetes and thyroiditis in recipient rats (Table 5.1). Levels of both acute diabetes and islet lesions were reduced in reconstituted rats when compared with non-reconstituted Tx-X controls ($P < 0.001$). In addition, incidence levels of thyroid lesions were dramatically reduced in reconstituted animals ($P < 0.001$) but TgAA levels were not affected (Table 5.1).

ii) Adoptive Transfer of Diabetes

Attempts to adoptively transfer diabetes using lymphoid cells from acute diabetic donors are summarized in Table 5.2. Although acute disease was not observed in any recipient, islet lesions were induced in a proportion (2/7) of normal 3 week old and adult irradiated (2/6) female SPF-derived recipients and in all (4/4) non-diabetic Tx-X recipients (Figure 5.1). In addition, all Tx-X recipients displayed abnormal glucose tolerance 10 days after cell transfer compared with a group of non-diabetic Tx-X controls maintained concurrently (Figure 5.2), suggesting these rats were more susceptible to transfer as a consequence of impaired immunoregulation.
Table 5.1. Effect of normal lymphocyte reconstitution on the development of autoimmune disease in Tx-X rats.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Reconstituted *</th>
<th>Control Tx-X</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>n</td>
<td>25</td>
<td>47</td>
</tr>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute Diabetes</td>
<td>1 (4)</td>
<td>10 (21)</td>
</tr>
<tr>
<td>Islet Lesions</td>
<td>2 (8) †</td>
<td>17 (36)</td>
</tr>
<tr>
<td>Thyroiditis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyroid Lesions</td>
<td>5 (20) §</td>
<td>28 (62)</td>
</tr>
<tr>
<td>TgAA (OD$_{405}$)</td>
<td>0.39 ± 0.08</td>
<td>0.41 ± 0.06</td>
</tr>
</tbody>
</table>

* Each received $1 \times 10^8$ viable syngeneic spleen cells IP 24 hours after final irradiation.

† $P < 0.01$ compared with controls.

§ $P < 0.001$ compared with controls.
Table 5.2. Incidence of acute diabetes, islet lesions and glucose intolerance in recipients of lymphoid cells from acute diabetic donors.

<table>
<thead>
<tr>
<th>Recipient Type *</th>
<th>Acute Diabetes</th>
<th>Islet Lesions</th>
<th>Glucose Intolerance /</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 weeks old</td>
<td>0 / 7</td>
<td>2 / 7</td>
<td>NT</td>
</tr>
<tr>
<td>6 weeks old</td>
<td>0 / 3</td>
<td>0 / 3</td>
<td>NT</td>
</tr>
<tr>
<td>Irradiated Adult †</td>
<td>0 / 6</td>
<td>2 / 6</td>
<td>NT</td>
</tr>
<tr>
<td>Tx-X §</td>
<td>0 / 4</td>
<td>4 / 4</td>
<td>4 / 4</td>
</tr>
</tbody>
</table>

* Each received 5 to 20x10^6 ConA-activated spleen cells from acute diabetic donors transferred IV.

† 1 x 2.5 Gray whole-body irradiation 1 day prior to transfer.

§ All recipients showed normal glucose tolerance prior to transfer. Control Tx-X rats (n=5) maintained concurrently showed no abnormalities.

/ As determined by IPGTT 10 days post-transfer.

NT = not tested.
Figure 5.1. Section of pancreatic islets of a recipient of diabetic lymphoid cells showing the onset of insulitis and islet atrophy (H&E; x 250)
Figure 5.2 IP glucose tolerance tests of Tx-X adoptive transfer recipients. Tx-X rats received 0.5 to 2.0x10^7 activated spleen cells IV from diabetic donors and were challenged IP 10 days later with glucose and blood glucose levels followed for the following 2 hours (open circles). Age-matched Tx-X rats (n=5) were used as controls (closed squares).
iii) Adoptive Transfer of Thyroiditis

In contrast to diabetes, thyroid lesions and thyroglobulin autoantibody production were readily transferred to both irradiated and normal recipients, but success was dependent upon the route of transfer and the cell dose used. Thus, transfer by the IV route was effective in inducing thyroid lesions and raised anti-Tg antibody levels in 11/15 (73 percent) of recipients (Figure 5.3; Table 5.3). In contrast, IP transfers failed to produce thyroiditis in any recipient animal.

The incidence and severity of thyroiditis in cell transfer recipients could also be modified by the number of cells used for transfer (Table 5.4). Thus, 5x10^7 activated thyroiditic spleen cells were capable of transferring disease to 4/4 normal PVG recipients with a mean pathology score of 2.0. Decreasing cell numbers resulted in a decline in incidence and severity of the lesion, with 2x10^7 cells inducing lesions in only 1 of 4 recipients with a mean pathology score of 1.0.

Transfers to irradiated recipients tended to follow the same pattern, except in the case of recipients of 4x10^7 cells, where 3/3 recipients developed thyroid lesions with a mean pathology score of 3.0, possibly due to the unintentional inclusion of a diabetic rat as a cell donor. Levels of anti-Tg autoantibodies tended not to be influenced by cell dose, remaining elevated in most cases (OD_{405} levels of 0.4 to 0.8) except for normal and irradiated recipients of 5x10^7 cells where antibody levels remained low.
Figure 5.3. Thyroid lesion of an adoptive cell transfer recipient of thyroiditic lymphoid cells showing cellular infiltration and loss of follicular integrity (H&E; x250).
Table 5.3. Influence of cell transfer route on the ability to adoptively transfer thyroiditis.

<table>
<thead>
<tr>
<th>Route of Transfer</th>
<th>Number of Transfers†</th>
<th>Incidence of Thyroiditis n (%)</th>
<th>Thyroid Pathology Score (x ± SEM)</th>
<th>TgAA (OD405) (x ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>IV</td>
<td>15</td>
<td>11 (73)</td>
<td>1.4 ± 0.2</td>
<td>0.6 ± 0.1</td>
</tr>
</tbody>
</table>

* Recipients were adult female normal PVG rats.

† Donors were Tg Ab+ and non-diabetic 10 week post-irradiation Tx-X rats. Rats received 20 x10^6 ConA - activated spleen cells per transfer.

P < 0.001 for all comparisons.
Table 5.4. Influence of cell-dose and recipient type on the adoptive transfer of thyroiditis.

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Cell Number Transferred (x10^6)</th>
<th>Incidence of Thyroiditis</th>
<th>Thyroid Pathology Score (x ± SEM)</th>
<th>TgAA (OD_{405}) (x ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Adult</td>
<td>50</td>
<td>4 / 4</td>
<td>2.0</td>
<td>0.2</td>
</tr>
<tr>
<td>PVG</td>
<td>40</td>
<td>4 / 5</td>
<td>1.4 ± 0.4</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>2 / 4</td>
<td>1.0 ± 0.6</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1 / 4</td>
<td>1.0</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Irradiated</td>
<td>60</td>
<td>5 / 5</td>
<td>1.4 ± 0.2</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Adult PVG</td>
<td>50</td>
<td>3 / 4</td>
<td>0.8 ± 0.2</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>40 §</td>
<td>3 / 3</td>
<td>3.0</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

* Donors were Tg AA⁺, non-diabetic 10 week post-irradiation Tx-X rats.

† Spleen cells from 4 to 5 donor rats were bulked prior to *in vitro* ConA activation and IV transfer.

§ Included 1 diabetic donor animal.
5.2.4 DISCUSSION

These studies have provided evidence of a role for cell-mediated immunity in the pathogenesis of Tx-X induced autoimmune disease, and give further support to the hypothesis that IDDM-induction by the Tx-X protocol is of an autoimmune origin.

Reconstitution of Tx-X rats with lymphoid cells from normal, syngeneic donors shortly after completion of the induction protocol significantly reduced the incidence of islet lesions (atrophy or insulitis) and lowered the incidence levels of acute diabetes (Table 5.1).

In addition, thyroid lesions were also significantly reduced in reconstituted animals (Table 5.1), an observation that is in accordance with a previous study on thyroiditis in this model (Penhale et al., 1976). Autoantibody production to thyroglobulin was not influenced by reconstitution in the present study, with both reconstituted and control animals producing significant levels of this antibody. In contrast, Penhale and associates did observe a reduction in antibody production to thyroglobulin, however this result may have been due to the relatively greater number of spleen cells used for reconstitution (4 x10^8 compared with 1x10^8 in the present study) (Penhale et al., 1976).

Together, these observations provide support for the following hypotheses: 1) The Tx-X procedure selectively depletes or inactivates particular subpopulations of T-lymphocytes and does not induce complete immunosuppression. This was demonstrated by observations in the present study that Tx-X animals were capable of significant autoantibody production to thyroglobulin (a thymus-dependent antigen) which was not
influenced by lymphocyte reconstitution, suggesting that sufficient T-helper and B-cell function were present to initiate this response. This is also supported by studies demonstrating the presence of substantial numbers of T and B cells in lymphoid organs of Tx-X animals (A. Stone, P. Stumbles and J. Penhale, unpublished observations); 2) The Tx-X procedure selectively modifies or depletes the suppressive activity of a population of T cells responsible for controlling potential cell-mediated autoreactivity in the normal animal. This is supported by the ability to restore this regulatory capacity by transfusions of lymphoid cells from normal animals.

Recent studies of T-cell reconstitution in Tx-X rats by Fowell and associates have identified the T-cell subset responsible for restoring immunoregulatory capacity in reconstituted Tx-X rats (Fowell et al., 1991). Reconstitution of Tx-X rats with thoracic duct lymphocytes fractionated on the basis of expression of the leukocyte common antigen (CD45) identified a T-cell of the CD45RC_{low}, CD4^+ phenotype capable of completely abrogating the expression of diabetes. In contrast, CD4^+, CD45RC_{high} T-cells had no suppressive effect, and were capable of inducing a severe wasting syndrome in congenitally athymic rats.

CD4^+, CD45RC_{low} T-cells have been shown to secrete low levels of IL-2 and IFN_γ and high levels of IL-4, and are therefore thought to represent a cell with so-called "Th2 - like" characteristics - that is, the ability to suppress cell mediated responses and to provide B cell help (Mason, 1992). In contrast, CD45RC_{high}, CD4^+ T cells secrete high levels of IL-2 and IFN_γ and appear to represent the "Th1" cell type involved in cell-mediated immune responses. The situation is complicated further however by heterogeneity within the CD45RC_{low} population based on expression of
other markers such as Thy-1, RT6 and OX-40 (Fowell et al., 1991), so at this stage the exact nature of the cell type involved is unclear. In addition, the interaction of these cells with other cell types is yet to be determined, particularly in relation to CD8\(^+\) T cells which have been shown to be essential for disease development in this model (Fowell et al., 1991).

Studies in the BB rat have also shown that the course of disease development can be altered by cellular reconstitution, as transfusion of whole blood from DR-BB rats eliminated the occurrence of diabetes in DP animals (Rossini et al., 1983). Subsequent studies showed that a CD4\(^+\), RT6\(^+\) T-cell was responsible for the suppression (Rossini et al., 1984; Mordes et al., 1987; Burstein et al., 1989).

Additional evidence for the pathological role of cell-mediated immunity in Tx-X-induced autoimmunity was provided by the ability to adoptively transfer disease to naive recipients using lymphoid cells from affected donors. Although acute diabetes could not be transferred using cells from diabetic donors, islet lesions were observed in a proportion of young normal and adult irradiated recipients (Table 5.2). Subsequent experiments on the adoptive transfer of thyroiditis using lymphoid cells from thyroiditic rats indicated that effective transfer of thyroid lesions could be achieved only if cells were transferred via the intravenous route (Table 5.3). Moreover, the severity of the lesion induced varied in a dose dependent manner, as transfer of 2\(\times\)10\(^7\) cells induced mild lesions, whereas doses of 4 to 5 \(\times\) 10\(^7\) produced strong lesions in all recipients (Table 5.4).

Thyroglubulin autoantibody production could also be adoptively transferred using cells from thyroiditic donors, although antibody titre
appeared to be independent of the number of cells transferred, being elevated to the same level in most cases regardless of the status of the recipient (irradiated or normal) (Table 5.4). The reason for this is unclear, but may indicate that autoantibody production can be adoptively transferred using relatively lower numbers of cells than that required for the transfer of lesions. The cell numbers used for transfer in this study were probably above the level required to observe a dose-dependent response for autoantibody production.

In light of these results, the inability to induce acute diabetes by intravenous transfer was probably due to the sub-optimal number of cells available from acute diabetic donors for transfer (0.5 to 2 x 10^7). In contrast, islet lesions were readily transferred to Tx-X recipients (Table 5.2) and in addition all recipients developed abnormal tolerance to IP glucose challenge (Figure 5.2). The enhanced susceptibility to disease transfer seen in Tx-X recipients probably reflected the immunocompromised state of these animals. Again however, acute disease was not observed in any recipient, again probably due to the low number of cells available for transfer. An alternative approach would be to identify and isolate lymphocytes for adoptive transfer from pre-diabetic Tx-X donors. Such cells would presumably be primed for B cell reactivity and may be more potent at transferring acute disease.

Several studies have demonstrated the ability to transfer diabetes in both BB rats and NOD mice. Adoptive transfer of diabetes in the BB rat was first reported in 1983 by Koevary and associates using ConA-activated spleen cells from acutely-diabetic BB rats transferred into young diabetes-prone animals (Koevary et al., 1983). In this situation however, successful transfer was assessed on the basis of accelerated disease onset in a
disease-prone animal and was therefore distinct from the present studies, where transfer was attempted into recipients not normally prone to disease. Subsequent studies showed that this effect could be mediated by CD4+ T cells (Metroz-Dayer et al., 1990). Similarly, ConA-activated spleen cells from acutely diabetic DP-BB rats were able to transfer disease to RT6-depleted DR recipients (Greiner et al., 1987).

In NOD mice, transfer of spleen cells from acutely diabetic animals to young (less than 6 weeks) NOD mice induced a higher frequency and earlier onset of symptoms than untreated littermates (Wicker et al., 1986a). Again however, successful transfer determined on the basis of inducing disease in an animal already susceptible to spontaneous disease, making the interpretation of these results difficult. Subsequent experiments have shown that transfer of disease is also possible into neonatal NOD animals or immunodeficient NOD-scid/scid mice, and demonstrate a requirement for both CD4+ and CD8+ T cells (Bendelac et al., 1987; Christianson et al., 1993).

In conclusion, these experiments provide strong evidence in favour of a pathological role for tissue-specific autoreactive T cells in the induction of autoimmune disease in Tx-X animals. The ability to transfer disease to naive recipients using activated lymphocytes from affected donors, in conjunction with the ability to suppress the onset of disease in Tx-X animals by transfusion of lymphocytes from normal animals, argued in favour of the conclusion that the Tx-X protocol selectively depletes or inactivates a population of T cells that act to suppress the activity of these potentially autoreactive cells in the normal animal. The characteristics of this suppressive population remain to be fully determined, but at this stage they appear to be CD4+ T cells of the CD45RClow, RT6+ phenotype.
5.3 IN VITRO CELL PROLIFERATION STUDIES

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5.3.1 INTRODUCTION

The observation that organ-specific autoimmune responses can be transferred to normal recipients using lymphoid cells from affected donors suggests a strong role for cell mediated autoimmune mechanisms in the pathogenesis of these diseases. Moreover, the fact that these autoreactive T cells are targeted to a specific tissue or cell type (for example, the β cell in IDDM) implies that these cells recognize and respond to an autoantigen(s) unique to the target tissue. It is thus of critical importance to identify these T-cell antigens as the first step in developing any specific therapeutic or prophylactic regime.

Using autoantibodies as probes, it has been possible to identify putative target autoantigens in a number of autoimmune diseases which may then be utilized in in vitro proliferation assays in conjunction with autoreactive T cells to assess their role in T-cell activation. Using this approach, recent studies have detected in vitro proliferation of lymphocytes from diabetic patients to islet cell autoantigens such as GAD and the 38kDa secretory granule protein, and also to non-islet antigens such as heat shock proteins, particularly HSP65, and dietary proteins such as bovine serum albumin (see section 2.4.2(i)).

This section describes attempts to identify reactivity of lymphoid cells from diabetic Tx-X rats to islet cell antigens in vitro, and to assess the potential role of previously-described autoantigens of other IDDM syndromes in the activation of autoreactive cells from these rats.
5.3.2 MATERIALS AND METHODS

i) Selection of Donors and Preparation of Cells

Non-diabetic Tx-X animals were selected on the basis of normal blood glucose readings and glucose tolerance. Acute diabetic donors were diabetic animals that had been stabilized by exogenous insulin therapy. Single spleen-cell suspensions were prepared as described in 5.2.2(ii).

ii) Preparation of Antigens and Mitogens

Cellular Antigens

Dispersed rat islet cells were prepared from intact islets isolated as previously described (see section 4.2.3). Islets were dispersed into single cell preparations by resuspending 1000 purified islets in 5ml of Ca\(^{2+}\)-free KRB-HEPES solution containing 0.5 percent bovine serum albumin (KRB-HEPES/BSA). After incubating for 1 minute at room temperature, the islets were centrifuged at 1800 rpm for 1 minute then resuspended in 1ml of KRB-HEPES/BSA containing 3mM EGTA. After 15 minutes at room temperature, islets were centrifuged and resuspended in 5ml of KRB-HEPES/BSA plus EGTA containing 0.1 percent trypsin (Difco 1:250, Michigan, USA), incubated for 5 minutes at room temperature with constant mixing then washed three times and resuspended in complete RPMI containing 10 percent FCS. Dispersed islet cells were allowed to recover overnight at 37°C in 5 percent CO\(_2\) then washed in complete RPMI-NRS and either used directly as antigen in proliferation assays (viable islets) or fixed in 1 percent paraformaldehyde (fixed islets) before being used as antigen.
Liver cells were prepared from rat liver by mincing the tissue and digesting with 1mg/ml collagenase Type V (Sigma Chemical Co., MO, USA) for 10 minutes at 37°C. The cells were then washed in HBSS-HEPES and resuspended in complete RPMI-NRS.

Heat Shock Proteins (HSP)

Recombinant Mycobacterium bovis HSP65 and M. tuberculosis HSP70 were kindly donated by the National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands. Lyophilized preparations were resuspended to 200 µg/ml in PBS, filter sterilized and stored at -20°C.

Bovine Serum Albumin (BSA)

BSA was purchased from Sigma Chemicals, MO, USA as a lyophilized powder. A 200 µg/ml stock solution was made in PBS, filter sterilized and stored at -20°C.

Mitogens

The T-cell mitogens ConA and PHA were prepared as described in section 5.2.2(i).

iii) Proliferation Assay Conditions

All proliferation assays were performed in complete RPMI containing 5 percent heat-inactivated normal rat serum (NRS). SpC suspensions were resuspended to 1x10⁶ cells per ml and 200µl (2 x10⁵ cells) added in
triplicate for each mitogen or antigen concentration to wells of 96 well round-bottom tissue culture plates (ICN Biomedicals, NSW, Australia). Cells were cultured at 37°C in 5 percent CO₂ for 72 hours with mitogens or 120 hours with antigen preparations.

iv) ³H-thymidine Labelling and Scintillation Counting

To assess cellular proliferation, 1µCi of ³H-thymidine (Amersham Int., Amersham, UK) in a volume of 5µl of RPMI was added to each well 18 hours prior to completion of the assay. At completion of the assay period cells were harvested onto glass-fibre filters using a Cambridge PHD® cell harvester (Cambridge Technology, MA, USA), dried overnight and dissolved in 2ml of scintillation cocktail (0.4 percent w/v Omnifluor [NEN Research, MA, USA], 33 percent v/v C:40 [Sigma Chemicals, WA, Australia], 67 percent v/v toluene). Counts per minute (CPM) were determined in a liquid scintillation counter (Tri-Carb® 4000, Packard Instrument Co., Illinois, USA) using a counting time of 3 minutes per tube. Mean CPM of triplicate wells are expressed as a stimulation index (SI) calculated as follows:

\[
SI = \frac{\text{Mean CPM stimulated cells}}{\text{Mean CPM unstimulated cells}}
\]

v) Statistical Analyses

Comparison of incidence rates was by chi-squared analysis of 2x2 contingency tables using the Yates' correction for n>50 or Fishers' Exact Test for n≤50 (Langley, 1971). Mean values were compared using Students' t-test for unpaired samples. Only significant figures are indicated.
5.3.3 RESULTS

i) Responses to Mitogens

In order to initially determine the proliferative capacity of lymphocytes from Tx-X animals, spleen-cell responses to a range of mitogens were assessed. A comparison of the proliferative responses from normal PVG, non-diabetic and diabetic Tx-X rat to the T cell mitogens ConA and PHA are shown in Figure 5.4. Normal PVG rat spleen cells showed a wide variation in responsiveness between individual animals to ConA and PHA, with SI values ranging from 60 to greater than 400 for ConA and 5 to approximately 200 for PHA (Figure 5.4a). Spleen cells from non-diabetic Tx-X animals showed an even broader range of responses to these mitogens, with SI values ranging from to 10 to greater than 450 for ConA and from 0 to 400 for PHA (Figure 5.4b). Note that overall, PHA responses of both normal PVG and TX-X rat cells were markedly reduced when compared to ConA values. Spleen cells from diabetic animals generally showed severely depressed responses to both ConA and PHA (Figure 5.4c).

The above mitogen-stimulation results are summarized in Table 5.5. Normal PVG PHA responses were significantly depressed compared with ConA values ($P <0.001$). Diabetic animals also showed significantly depressed responses when compared with normal Tx-X and normal PVG rats ($P <0.02$ and 0.001 respectively). Note that there was no significant difference between ConA or PHA responses of normal Tx-X or PVG animals. In addition, Tx-X animals showed depressed B cell mitogenic activity as indicated by a reduced responsiveness to PWM compared with normal levels ($P <0.02$).
Figure 5.4. Proliferative responses of normal and Tx-X rat spleen cells to ConA and PHA. Spleen cells were isolated from normal PVG (a), clinically normal Tx-X (b) and acute diabetic (c) rats, cultured for 72 hours with ConA or PHA (5µg/ml) and proliferation assessed by ³H-thymidine incorporation. Symbols represent individual animals.
Table 5.5. Mitogen-induced proliferative responses of Tx-X and normal PVG rat spleen cells.

<table>
<thead>
<tr>
<th>Spleen Cell Source</th>
<th>ConA *</th>
<th>PHA</th>
<th>PWM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal PVG</td>
<td>207 ± 24 †</td>
<td>64 ± 13</td>
<td>53 ± 8</td>
</tr>
<tr>
<td>(n=17)</td>
<td>(n=14)</td>
<td></td>
<td>(n=5)</td>
</tr>
<tr>
<td>Tx-X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>140 ± 44 §</td>
<td>33 ± 6</td>
<td>19 ± 7 //</td>
</tr>
<tr>
<td>(n=12)</td>
<td>(n=11)</td>
<td></td>
<td>(n=5)</td>
</tr>
<tr>
<td>Acute Diabetic</td>
<td>38 ± 9</td>
<td>21 ± 11</td>
<td>-</td>
</tr>
<tr>
<td>(n=14)</td>
<td>(n=9)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean SI ± SEM

* ConA and PHA were used at 5 µg/ml. PWM was used at 1 µg/ml.

† $P < 0.001$ compared with normal PVG PHA and diabetic ConA values.

§ $P < 0.02$ compared with diabetic ConA value.

// $P < 0.02$ compared with normal PVG PWM value.
The similarities and differences in lymphocyte mitogen responses between the above groups of animals were further highlighted by mitogen dose-response curves (Figure 5.5). Although normal Tx-X animals occasionally showed spleen-cell ConA dose responses similar to those of normal PVG animals, these were observed to be highly variable as illustrated in Figure 5.4, where some rats showed proliferative responses of an SI of 50 or less. Similarly, PHA responses of normal Tx-X rats were variable but generally depressed, tending to resemble those of diabetic animals, reaching a peak SI value of only 25 compared with values greater than 100 for normal PVG animals.

In contrast, diabetic animals showed consistently depressed proliferative responses to both ConA and PHA at all doses, reaching SI peaks of approximately 50 and 25 respectively (Figure 5.5). Note that high doses of mitogen (50µg/ml) appeared to be inhibitory for all groups, and that overall proliferative responses to PHA were depressed for each group of rats for all mitogen doses.

**ii) Responses to Antigens**

To detect lymphocyte reactivity to various antigens implicated in IDDM, spleen cells from normal and Tx-X animals were used in proliferation assays in conjunction with a range of cellular and soluble antigens.

Initially, to determine whether cellular autoreactivity to islet cells could be demonstrated in Tx-X animals, spleen cells from acute diabetic, normal Tx-X or normal PVG rats were co-cultured with purified rat islet cells (Figure 5.6). Low levels of reactivity to viable islet cells were observed
Figure 5.5 72 hour proliferative responses of spleen cells isolated from normal PVG (closed circles; n=8), clinically normal Tx-X (open squares; n=4) and acute diabetic rats (closed squares; n=9) to various doses of ConA and PHA.
Figure 5.6 Proliferative responses of normal and Tx-X rat spleen cells to islet cell antigens. Spleen cells from normal PVG (a) and Tx-X (b) rats were cultured for 72 hours in the presence of viable or formalin-fixed dispersed islet cells, or viable liver cells as controls, and proliferation assessed by $^3$H-thymidine incorporation.

Symbols represent individual animals.

Open squares indicate acute diabetic rats.
both with normal spleen cells (Figure 5.6a) and Tx-X spleen cells (Figure 5.6b) with no differences in levels of reactivity between acute diabetic and non-diabetic animals.

In contrast, no reactivity was observed towards fixed islet cells (Figure 5.6), indicating that either 1) a stimulatory factor may have been present in viable islet cell cultures; 2) that the fixation process destroyed any autoantigenic epitopes, or 3) that potential islet autoantigens were unable to be processed and presented by dedicated antigen presenting cells.

To determine whether an HSP, particularly HSP65, may act as potential autoantigens in the Tx-X syndrome, cellular reactivity towards recombinant HSP65 and also HSP70 was assessed (Figure 5.7). A small degree of reactivity was observed only to HSP70, where one normal PVG animal showed an SI value of 25 (Figure 5.7a) and one normal PVG and one diabetic animal responded at lower levels (Figure 5.7a and b). In contrast, no reactivity was observed to either HSP65 or BSA in normal or Tx-X rats.

5.3.4 DISCUSSION

Although it is clear that cell-mediated immune mechanisms are important in the pathogenesis of Tx-X induced diabetes, lymphoid cells from Tx-X animals generally showed poor proliferation in vitro.

Initially, in order to investigate the overall status of T cell reactivity of Tx-X animals, in vitro spleen cell responses to T cell mitogens were examined.
Figure 5.7. Proliferative responses of spleen cells from normal and Tx-X rats to soluble antigens. 2x10^5 spleen cells from normal PVG (a) and Tx-X (b) rats were cultured for 72 hours in the presence of 10µg/ml of each antigen.

Symbols represent individual animals.

Open squares indicate acute diabetic rats.
As expected, spleen cells from non-diabetic Tx-X animals showed reduced proliferation to ConA when compared to spleen cells from normal rats (Figures 5.4 and 5.5, Table 5.5). Furthermore, spleen cells from acute diabetic animals showed no significant proliferation to ConA, indicating a severe impairment of T cell function in these animals. The proliferation of spleen cells from rats of all groups to PHA were generally low, thus indicating this to be a poor rat T-cell mitogen.

Attempts to characterize the target autoantigens of autoreactive T cells in Tx-X rats by in vitro lymphocyte proliferation proved inconclusive. In order to investigate potential β cell antigens in vitro, it was considered necessary initially to demonstrate that lymphocytes from diabetic Tx-X rats would proliferate in response to intact, viable β cells. Although low levels of proliferative activity were observed, this was matched by similar low levels of activity in normal animals, thus questioning the disease specificity of the response. These results, however, appear to be consistent with findings in the BB rat, where there have been no reports of T-cell reactivity to any islet cell antigens. Nevertheless, this does contrast with studies in humans and NOD mice, where there have been small number of reports of T cell reactivity to islet antigens.

In humans, Harrison et al. reported the detection of T cells in peripheral blood reactive to human islets and pig pro-islets (Harrison et al., 1991a). This group has also reported low levels of T cell reactivity towards sonicates of foetal pig pro-islets in preclinical patients, and this reactivity appeared to correlate well with ICA levels and progression to clinical disease (Harrison et al., 1992a). There have also been a small number of reports of CD4+ and CD8+ T cell lines and clones generated to islet cell antigens (DeBerardinis et al., 1988; Konttinen et al., 1991).
To date, there has only been one report of *in vitro* T cell reactivity to islet antigens in NOD mice (Burtles et al., 1992). These workers also demonstrated a similar degree of islet-cell reactivity in non-diabetes prone mice strains, suggesting that lack of tolerance to islet cell antigens may be a common feature of mice and that diabetes-development in NOD mice may be a result of a dysfunction in the regulation of these autoreactive cells. The difficulty in demonstrating *in vitro* T cell reactivity to islet antigens may be the result of a combination of several factors. Firstly, autoreactive T cells are probably present at low frequency and any proliferative response may not be detected under standard assay conditions. Secondly, the islet cell target antigen may only be transiently expressed under normal circumstances and may be particularly sensitive to degradation *in vitro*. Thirdly, appropriate presentation of antigen to T cells may not occur under *in vitro* conditions.

Similarly, there have been few reports of *in vitro* T cell reactivity to purified islet cell antigens. Atkinson et al. reported peripheral blood lymphocyte responses to recombinant GAD65 in 67 percent of newly diagnosed IDDM patients and ICA+ relatives (Atkinson et al., 1991) and Roep et al. demonstrated T cell reactivity to the 38kDa insulin-secretory granule protein in 80 percent of recent-onset patients, and were able to generate T cell clones to this antigen (Roep et al., 1991). In NOD mice, Elias et al. reported T cell reactivity to the 65kDa heat shock protein (HSP65), but not to HSP70 (Elias et al., 1990). In addition, epitopes of the HSP65 molecule could be used to vaccinate against diabetes development (Elias et al., 1991). These results contrast with the present study, where no T cell reactivity was observed towards HSP65 whereas low levels were detected towards HSP70 in both Tx-X and normal animals.
In conclusion, the ability to transfer disease to normal syngeneic recipients using lymphoid cells isolated from affected Tx-X rats demonstrates that islet-reactive T cells are generated during the diabetic syndrome and demonstrates an essential role of cellular immune mechanisms in the pathogenesis of diabetes and thyroiditis induced by this procedure. Although islet-reactive T cells could be demonstrated in Tx-X rats that were capable of transferring disease, demonstration of this reactivity *in vitro* was difficult to achieve. Further work is required to determine the appropriate form of the antigen and the appropriate assay system for detecting the potentially low levels of T cell responses. The ability to suppress disease onset by reconstituting Tx-X animals with normal lymphoid cells supports the concept that maintenance of self-tolerance under normal circumstances is achieved in part by an active suppressive mechanisms and that the Tx-X procedure disrupts this immuno-regulatory mechanism. Finally these studies illustrate the value of this model for such studies in that normal, syngeneic animals are readily available for the provision of cells and as recipients for cell transfer studies.
5.4  IN VITRO MACROPHAGE DEPLETION STUDIES

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5.4.1 INTRODUCTION

Although there is strong evidence in support of an important role for T cells in the pathogenesis of Tx-X-IDDM, the in vitro responses of spleen cells from diabetic Tx-X rats, and to a lesser extent non-diabetic rats, were characterized by poor proliferation in response to T-cell mitogens (see previous section). While this apparent paradox could be explained by the poor condition of cells from clinically diabetic animals, or the inability of these cells to produce appropriate growth factors, the poor responsiveness of spleen cells from clinically normal Tx-X animals in comparison to normal PVG rats raised the possibility that the proliferation of these cells was being suppressed in vitro.

The phenomenon of in vitro suppression of lymphocyte proliferation has been observed in a number of experimental models of autoimmune disease, including both BB rats and NOD mice. In both these cases, a notable improvement in mitogen-induced proliferation was observed if spleen cell cultures were depleted of adherent cell populations prior to activation (Prud'homme et al., 1984; Woda and Padden, 1986; Yokono et al., 1989). For BB rats, removal or inactivation of macrophages significantly improved spleen cell proliferative responses to ConA and IL-2 production, while adherent cells from diabetic rats could inhibit normal MHC-matched control cells (Prud'homme et al., 1984). In addition, Metroz-Dayer and associates showed that the removal of macrophages prior to in vitro ConA stimulation resulted in an enhanced incidence of diabetes in adoptive transfer recipients of spleen cells from diabetic NOD mice (Metroz-Dayer et al., 1990). Moreover, the presence of suppressor macrophages has also been described in other autoimmune diseases.
such as rheumatoid arthritis and systemic lupus erythematosis (Zembda and Lemmel, 1980; Sieper et al., 1992).

The suppressive ability of macrophages has been recognized for some time (see Allison, 1978 and Unanue and Allen, 1987 for reviews). Initial studies showed that addition of peritoneal macrophages to rabbit spleen cell cultures could inhibit spleen cell DNA synthesis in response to antigen (Parkhouse and Dutton, 1966). Subsequent studies in rats also described this effect for allogeneic mixed lymphocyte reactions (Folch and Waksman, 1974; Oehler et al., 1977; Hoffman et al., 1990) and spleen cell responses to mitogens (Folch et al., 1973; Webb et al., 1980). It appears that macrophages isolated from other sites are also capable of *in vitro* cytostatic activity, as this effect has also been described for alveolar macrophages (Holt, 1979) although there is some evidence to suggest that the type of macrophage capable of suppression is species-dependent (Holt, 1980).

As a result of the above observations, and in an attempt to determine the basis for the poor *in vitro* proliferative responses to mitogens and antigens of lymphocytes from Tx-X rats, studies were undertaken to determine whether there was a role for suppressor macrophages in Tx-X-induced IDDM.
5.4.2 MATERIALS AND METHODS

i) Selection of Cell Donors

Tx-X spleen cell donors were either acute diabetic rats with recent-onset acute diabetes that had been stabilized with insulin, or normal rats showing normal blood glucose levels and IP glucose tolerance. Age-matched non-Tx-X PVG rats (normal PVG) were used as a source of normal cells.

ii) Depletion of Splenic Macrophages

Single-cell spleen suspensions were prepared as previously described (section 5.3.2(i)) and depleted of macrophages by plastic-adherence (Hunt, 1987). Washed cell suspensions were resuspended to 1 to 2 x 10^6 cells/ml in complete RPMI containing 5 percent NRS (RPMI/NRS), and 5 to 7 ml plated onto 60mm tissue-culture grade plastic dishes (Falcon® 3002; Becton Dickinson, NJ, USA) and incubated for 2 hours at 37°C in 5 percent CO₂. The non-adherent cells were then harvested and used directly in subsequent proliferation assays.

iii) In Vitro Proliferation Assay Conditions

Triplicate wells of 96 well tissue culture plates were established for each assay condition using 2x10^5 responder cells per well in a volume of 200μl of complete RPMI/NRS. ConA was added to each well at a final concentration of 5 μg/ml and all cultures were incubated for 72 hours at 37°C in 5 percent CO₂. In some cases PHA and PWM were used at concentrations of 5μg/ml and 1μg/ml respectively. ³H-thymidine labelling
and cell harvesting were performed as previously described (section 5.3.2(iv)). Results are expressed as a stimulation index (SI) calculated as follows:

\[
SI = \frac{\text{mean CPM ConA-stimulated responder cells (+/- adherent cells)}}{\text{mean CPM non-depleted responder cells alone}}
\]

5.4.3 RESULTS

j) Influence of Macrophage Depletion In Vitro

Responses to Mitogens

To examine the possible influences of macrophage depletion on the in vitro activity of lymphocytes from Tx-X rats, initial experiments were performed to compare the mitogen-induced responses of adherent cell-depleted cultures compared to whole spleen cell cultures from Tx-X or normal PVG animals (Table 5.6).

In response to ConA, macrophage depleted Tx-X spleen cells showed markedly improved proliferation (mean SI of 219 compared with 31 in controls; \( P <0.02 \)) and this was also observed to a lesser extent in response to PWM (mean SI of 52 compared with 19 in controls) when compared to non-depleted Tx-X spleen cell controls. In contrast, macrophage depletion had no significant effect on normal PVG spleen cells for all mitogens (Table 5.6).
Table 5.6. Influence of adherent-cell depletion on mitogen-induced proliferative responses of Tx-X and normal PVG spleen cells.

<table>
<thead>
<tr>
<th>Mitogen</th>
<th>Tx-X (n=5)</th>
<th>Normal PVG (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-depleted</td>
<td>Depleted</td>
</tr>
<tr>
<td>ConA</td>
<td>31 ± 6</td>
<td>219 ± 65 *</td>
</tr>
<tr>
<td>PHA</td>
<td>37 ± 9</td>
<td>43 ± 13</td>
</tr>
<tr>
<td>PWM</td>
<td>19 ± 7</td>
<td>52 ± 14</td>
</tr>
</tbody>
</table>

Values are mean SI ± SEM

* P < 0.02 compared with "+ adherent cell" value
The mitogenic responses of spleen cells from individual animals in the presence or absence of adherent macrophages are shown in Figure 5.8. For Tx-X animals, removal of adherent cells resulted in a uniform improvement in the proliferative responses to ConA and PWM in all cases (Figures 5.8b and f respectively), whereas there was little or no improvement in proliferation to PHA after macrophage depletion (Figure 5.8d). In contrast, for normal PVG animals, there were no significant differences in the responses of depleted and non-depleted spleen cell cultures to the mitogens tested, although there was a considerable degree of individual variation, particularly for PHA and PWM responses (Figures 5.8a,c,e).

Disease Association

In an attempt to quantify the suppressive influence of adherent cell populations on the mitogenic responses of lymphocytes, and to examine any disease-related effects, in vitro adherent-cell depletion and reconstitution studies were undertaken.

Initially, splenic adherent cells were removed from diabetic rat spleen cell cultures and their proliferation to ConA compared with those of spleen cells isolated from clinically normal Tx-X animals (Table 5.7). For diabetic rats, macrophage depletion increased spleen cell proliferation to ConA by approximately 100 percent, although this was not statistically significant. In non-diabetic Tx-X animals, macrophage depletion resulted in a statistically significant increase ($P < 0.05$).
Figure 5.8. Influence of macrophage depletion on the proliferative responses of spleen cells. Suspensions of normal PVG (a,c,e) or clinically normal Tx-X (b,d,f) spleen cells were depleted of macrophages by plastic adherence, then activated with ConA, PHA (5µg/ml) or PWM (1µg/ml) for 72 hours and proliferation determined by ³H-thymidine incorporation.
Table 5.7. Response of spleen cells from acute diabetic or clinically normal Tx-X rats following macrophage depletion.

<table>
<thead>
<tr>
<th>Adherent-cell Status</th>
<th>Acute Diabetic (n=5)</th>
<th>Clinically Normal (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-depleted</td>
<td>48 ± 15</td>
<td>83 ± 23</td>
</tr>
<tr>
<td>Depleted</td>
<td>83 ± 38</td>
<td>185 ± 44 *</td>
</tr>
</tbody>
</table>

Values are mean SI ± SEM

* P < 0.05 compared with SI value of non-depleted cultures.
From this data it appeared that the adherent cell populations of diabetic spleen cell cultures were suppressive. To assess whether the *in vitro* suppressive capacity of splenic macrophages could be related to the disease status of the animal, normal PVG rat adherent-cell depleted spleen cell cultures were reconstituted *in vitro* with splenic adherent cells from either acute diabetic or clinically normal Tx-X rats (Figure 5.9). Adherent cells from diabetic animals did appear to have suppressive activity as they were capable of suppressing the proliferation of normal spleen cells to ConA (Figure 5.9a). The affect did not appear to be disease-related however, as adherent cells from clinically normal Tx-X rats suppressed the responses to the same degree (Figure 5.9b).

**ij) Adoptive Cell Transfers**

In view of the improved *in vitro* proliferative responses of macrophage-depleted spleen cell cultures to ConA, studies were undertaken to determine if this increased activation could enhance the ability to adoptively transfer disease to naive recipients using spleen cells from diabetic animals.

Spleen cells isolated from acute diabetic rats were depleted of adherent-cells then activated *in vitro* with ConA prior to IV transfer to 3-week old normal PVG rats (Table 5.8). Although neither acute disease or abnormal glucose tolerance were observed in recipient animals of spleen cells from acute diabetic rats, all developed islet lesions involving mild atrophy. This was an improvement on the results obtained when non-adherent cell depleted spleen cells were used, where only 2/7 (29%) of recipients developed islet lesions (see Table 5.2), indicating that
Figure 5.9. *In vitro* reconstitution of normal spleen cell cultures with Tx-X-derived adherent cells. Adherent-cell depleted spleen cell cultures from normal rats were reconstituted with adherent cells from either acute diabetic (a) or clinically normal (b) Tx-X rats, then cultured with ConA (5μg/ml) for 72 hours and proliferation assessed by $^3$H-thymidine incorporation.
Table 5.8. Incidence of acute diabetes, glucose intolerance and islet lesions in recipients of ConA-activated, macrophage depleted spleen cells isolated from Tx-X and normal donors.

<table>
<thead>
<tr>
<th>Cell Donors</th>
<th>No. of Transfers</th>
<th>Acute Diabetes</th>
<th>Glucose Intolerance</th>
<th>Islet Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute Diabetic</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3§</td>
</tr>
<tr>
<td>Normal Tx-X †</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Normal PVG</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>?//@</td>
</tr>
</tbody>
</table>

* 3 week-old normal PVG recipients received 1-4x10⁷ spleen cells IV depleted of adherent cells prior to in vitro ConA stimulation.
† Selected on the basis of normal blood glucose and glucose tolerance prior to transfer.
§ Lesions were classified as atrophy and insulitis.
//@ 2 animals developed mild islet changes.
adherent-cell depletion did improve the mitogenic activation of autoaggressive cells. Nevertheless, the continued inability to transfer signs of acute disease suggests that the adoptive transfer conditions had still not been optimized, possibly as a result of low numbers of cells again being available for transfer.

In contrast, no recipients of ConA-activated, adherent-cell depleted spleen cells from normal Tx-X or normal PVG animals developed islet lesions, or though there was some suggestion of mild islet change in 2 recipients of normal PVG cells (Table 5.8). The latter, although recorded, was considered to be an artefact of histological preparation.

5.4.4 DISCUSSION

The above studies have provided evidence that the Tx-X procedure, in addition to enhancing the activity of autoaggressive cells, is also enhancing the activity of mechanisms capable of suppressing the normal activity of lymphocytes in vitro. The above depletion studies suggest that this suppressive activity appears to be a function of adherent macrophage populations.

Removal of adherent macrophages from spleen cell cultures of Tx-X rats significantly improved the proliferative response to ConA and to a lesser extent PWM, a phenomenon not observed for spleen cells isolated from normal (non-Tx-X) rats (Table 5.6). The failure to improve mitogen responsiveness to PHA may possibly be due to this being a poor rat T-cell mitogen. This improved mitogen-induced proliferation was a consistent feature of Tx-X rats, restoring proliferation to levels equivalent to those of
normal animals in most cases (Figure 5.8) and argues in favour of increased activity or numbers of suppressive adherent cells in Tx-X rats compared to normal animals.

These findings extend those of several studies describing suppressor macrophages in rat spleen (Parkhouse and Dutton, 1966; Folch and Waksman, 1974; Oehler et al., 1977; Webb et al., 1980). Particularly relevant are studies of the BB rat and NOD mouse models of IDDM, where removal of adherent cells from spleen cell cultures markedly improved the mitogen responsiveness of these cells (Woda and Padden, 1986; Yokono et al., 1989) and allowed increased synthesis of IL-2 (Prud'homme et al., 1984). Although not examined in this study, preliminary evidence suggests that macrophage depletion also improves IL-2 secretion by Tx-X rat spleen cells in response to ConA.

Having established that increased macrophage-suppressive activity was a consistent feature of Tx-X animals, it was of interest to determine whether this phenomenon could be related to the occurrence of diabetes in Tx-X animals. Thus, spleen cells from diabetic and non-diabetic Tx-X animals were compared for improved proliferative capacity to ConA after removal of their adherent cells. As shown in Table 5.7, removal of adherent cells from spleen cell cultures of diabetic rats improved the ConA-induced proliferative responses, however this improvement did not reach statistical significance and was not as pronounced as that seen for non-diabetic Tx-X animals.

The suppressive activity of splenic macrophages isolated from diabetic animals was further demonstrated by their ability to substantially inhibit the ConA-induced proliferation of normal spleen cells, although the degree of
suppression was similar to that seen with clinically normal Tx-X animals (Figure 5.9). Overall, this data suggests that macrophages isolated from diabetic animals do have considerable suppressive activity, however the degree of suppressive activity did not correlate with the occurrence of acute disease, being similar in both affected and unaffected Tx-X animals.

Finally, there was an indication that macrophage-depletion of spleen cells from Tx-X rats prior to ConA activation and adoptive transfer improved the ability to transfer islet lesions, presumably as a result of improved T-cell activation (Table 5.8). These results are consistent with those of Metroz-Dayer et al., who demonstrated a similar phenomenon in the BB rat (Metroz-Dayer et al., 1990), and thus consistent with the hypothesis that macrophage depletion leads to improved T-cell activation by mitogens in the rat.

A number of mechanisms have been proposed to explain the molecular basis of macrophage suppression, including a role for prostaglandins, hydrogen peroxide and soluble protein messengers (Metzger et al., 1980; Unanue and Allen, 1987; Hoffman et al., 1990). Macrophage suppressive activity, however, appears to require activated macrophages (Metzger et al., 1980; Hoffman et al., 1990) and in the Tx-X model, this is supported by immunohistochemical studies demonstrating increased numbers of activated macrophages (la+ in both the spleen and other lymphoid organs such as peripheral lymph nodes and gut-associated lymphoid tissue (Stone and Penhale, unpublished observations). Macrophages may be activated by a number of signals, including microbial antigens, immune complexes, complement components and cytokines such as IFNγ (Allison, 1978; Albina et al., 1991).
Recently nitric oxide (NO), a product of L-arginine metabolism via the nitric oxide synthetase pathway, has emerged as the favoured mediator of lymphocyte suppression by activated rat macrophages. Several studies have shown that the inhibition of NO synthesis by NG-monomethyl-L-arginine (NGMMA), an inhibitory analogue of L-arginine, augments the response of rat lymphocytes to ConA (Albina et al., 1991; Mills, 1991; Kawabe et al, 1992) and to alloantigens (Hoffman et al., 1990). Of particular interest is that IFNγ, a product of activated Th1-type, CD4+ T-cells stimulates the NO synthesis pathway of macrophages, while IL-4, a product of Th2-type T-cells, is a potent inhibitor of this pathway (Albina et al., 1991; Al-Ramadi et al., 1992; Appelberg et al., 1992).

In addition to its suppressive effects of on lymphocyte proliferation, NO synthesis by islet β cells has also been shown to be mediator of IL-1β-induced inhibition of insulin secretion and β cell destruction (Corbett et al., 1992). The effect was shown to β cell specific, as IL-1β did not affect the metabolic activity of islet α cells. Furthermore, NGMAA has been shown to prevent the non-specific destruction of islet cells by activated macrophages (Kroncke et al., 1991) and the IL-1β-induced inhibition of glucose-stimulated insulin secretion by islets (Southern et al., 1990).

In conclusion, the above data is consistent with the hypothesis that the Tx-X procedure is enhancing the activity of a population of cells that, at least in the spleen, are capable of suppressing normal lymphocyte function, possibly via the actions of NO. This presents somewhat of a paradox, however, as data from this and previous sections have demonstrated a strong role for cell mediated immune mechanisms in the pathogenesis of Tx-X induced disease. Thus, the possibility arises that the suppressive activity of macrophages in Tx-X rats is acting on T-cells in a selective
manner, antagonizing the actions of "Th2" type suppressive cells, while promoting the activity of "Th1" type effector cells. At present there is no evidence that NO has such selective inhibitory activity, however the questions of whether this suppressive activity is mediated by NO, and the possible role of increased NO production \textit{in vivo} in the pathogenesis of disease, are currently being investigated.
CHAPTER 6.0
ORAL TOLERANCE STUDIES
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6.1 INTRODUCTION

As shown previously, a striking feature of autoimmune diabetes and thyroiditis induced by the Tx-X procedure was the high degree of variability in the incidence levels of these diseases seen between successive groups of animals (see chapter 3.0, Figure 3.7). Why should there have been such variability given that the PVG strain is highly inbred and all animals should therefore have been genetically identical? Although random rearrangement of T cell receptor elements could have accounted for a small degree of individual variability, it is unlikely that this could have accounted for such striking differences in the incidence of disease between successive groups. Thus, identification of this non-genetically determined factor(s) capable of influencing the course of disease to such a profound degree could provide a major step towards unravelling the complex aetiology of this and other autoimmune syndromes.

For some time the role of environmental factors in the pathogenesis of autoimmune disease has been debated (see chapter 2.4.3). Although environmental factors may take a number of forms and may influence the animal via many pathways, it is probable that the greatest opportunity for diversity is that exerted by the variability of antigenic challenge via the gastrointestinal (GI) tract. The mucosal surfaces of the GI tract are constantly encountering substances capable of modifying or stimulating the immune system, and as a result mucosal immunity must involve a closely regulated system that allows selective reactivity to pathogenic material while remaining tolerant to the vast array of non-harmful or essential molecules.
The lymphoid tissues of the GI tract include organized structures (Peyer's patches) consisting of large lymphoid follicles and associated epithelium that constitute the major antigen processing system, in association with many isolated lymphoid follicles dispersed throughout the submucosae (Keren, 1992). The lymphoid follicles of the gut contain a large number of B lymphocytes that are precursors for plasma cells generating IgA, and this is the primary immunoglobulin isotype found within the intestinal lumen (Biewenga et al., 1993). Nevertheless, both IgG and IgM-containing plasma cells are present in the gut, and there is some evidence to suggest that the production of either IgA or IgG and IgM may provide a means by which the mucosal immune system is activated towards a positive (immunizing) or suppressive (tolerizing) response to an antigen (Strober et al., 1981). In addition, CD4+ and CD8+ T lymphocytes are also present throughout the GI tract, located primarily interepithelially or within the lamina propria (Keren, 1992), and these undoubtedly play a key regulatory role, although precisely how remains uncertain at this stage.

The ability to manipulate the immune system via the gastrointestinal route has been well documented. Early studies by Vaz et al. showed that mice given a single dose of ovalbumin (OVA) were immunologically tolerant to OVA, producing low levels of IgG and IgE anti-OVA antibodies after subsequent immunization with the antigen (Vaz et al., 1978). Other studies have shown that animals may also be immunized to OVA by the oral route, depending on the dosage regime and the age of the animal (Faria et al., 1993). In addition, T and B cells have been shown to be important in regulating tolerance to orally encountered OVA (Peng et al., 1989).
For animal models of autoimmune disease where the autoantigen has been defined, oral administration of antigen has been shown to be protective. Probably the most extensively studied model in this respect is experimental allergic encephalomyelitis (EAE) induced in Lewis-strain rats by immunization with myelin basic protein (MBP). Higgins and Weiner demonstrated that the oral administration of MBP suppressed the development of EAE (both clinically and histologically) and also the *in vitro* proliferative responses of T cells to MBP and production of anti-MBP antibodies (Higgins and Weiner, 1988).

Studies on EAE are of particular interest as recently trials have begun to assess the efficacy of oral administration of MBP for the treatment of multiple sclerosis (Weiner et al., 1993). These studies of course assume that the mechanism of oral tolerance is operating effectively in MS patients. Clearly, if this is not the case, then the administration of MBP could lead to enhanced immune stimulation and perhaps more serious disease.

Oral administration of autoantigen has also been used to suppress the onset of disease in other models of autoimmunity, including S-antigen in experimental autoimmune uveitis (Thurau et al., 1991) and type-II collagen in collagen-induced arthritis (Thompson and Staines, 1985; Nagler-Anderson et al., 1986). For IDDM, there has been one report of the suppression of diabetes onset in NOD mice by oral administration of insulin over a one year period from 5 weeks of age (Zhang et al., 1991) and in streptozotocin (STZ)-induced diabetes by the oral administration of islet tissue before and during STZ treatment (Kolb et al., 1991).
Despite the large number of studies of oral tolerance, its mechanism is still unclear. For soluble proteins such as OVA and MBP, suppressor T cells have been implicated, as shown by the ability to adoptively transfer suppression by lymphocytes or to abrogate tolerance by immunosuppressive agents such as cyclophosphamide (Richman et al., 1978; Lider et al., 1989). For MBP, others have proposed that antigen feeding induces clonal anergy rather than suppressor cells (Whitacre et al., 1991), while in other systems, suppressor B cells have been implicated (Asherson et al., 1977) and in some cases serum factors have been reported to transfer tolerance, implicating a role for antibody or immune-complexes in tolerance induction (Andre et al., 1975).

For Tx-X-induced autoimmune disease, evidence of a role for gastrointestinal-derived antigens in the pathogenesis of disease has already been provided in this study by the inability to induce IDDM in conventionally-reared animals, presumably differing from SPF animals in their gastrointestinal bacterial load (see section 3.3.2(ii)), and in a previous study by the ability to augment the susceptibility of SPF animals to thyroiditis by feeding gastrointestinal contents derived from conventional animals (Penhale and Young, 1988). Thus, it appears that intestinal-derived factors have the potential to profoundly modify the susceptibility of Tx-X rats to autoimmune disease, acting either to enhance or decrease susceptibility, possibly as a result of abnormalities in immune tolerance towards gastrointestinal-derived antigens in these animals.

The following chapter describes a set of preliminary studies undertaken to assess the immune status of Tx-X animals to antigens processed via the gastrointestinal route. Thus, the influence of feeding a soluble protein antigen (OVA) on the subsequent immune response to systemic antigenic
challenge was assessed, both in terms of cell-mediated and humoral immune responses.

6.2 MATERIALS AND METHODS

6.2.1 Induction of Oral Tolerance

A schematic representation of the protocol used for inducing oral tolerance to ovalbumin (OVA) in Tx-X rats is shown in Figure 6.1. Tx-X rats for OVA feeding were chosen on the basis of normal blood glucose levels and normal glucose tolerance 8 weeks after final irradiation. Age-matched, normal female PVG rats were used as controls. Fed animals were given four 100mg doses of OVA (Fluka Chemicals, Buchs, Switzerland) in 0.5ml of bicarbonate buffer (PBS containing 0.15M NaHCO₃, pH 7.4) at 2 day intervals. Doses were given directly into the stomach under ether anaesthesia using a blunted 23 gauge needle and plastic tubing. Three days following the final feed, all animals were immunized with 200µg of OVA in Complete Freund's Adjuvant injected into the right hind foot pad. Blood samples were taken at 0, 9, 15, 22 and 29 days following feeding, and the animals killed 36 days following the first OVA feed.

6.2.2 Assessment of Immunological Tolerance

i) Delayed-type Hypersensitivity (DTH)

Prior to killing, all animals were tested for DTH responses to OVA by ear skin-testing. 20 µg of OVA in 0.05ml of PBS was injected intradermally
Figure 6.1. Protocol for the induction and assessment of oral tolerance to OVA in Tx-X rats. Tx-X or normal PVG rats were fed four 100mg doses of OVA 60 days after final irradiation, then immunized with 200µg of OVA three days later. Bleeds were taken at various times during the experiment and analyzed for anti-OVA antibody, and DTH and in vitro proliferative responses to OVA assessed three weeks after the first feed.
into one ear of each rat and an equivalent volume of saline injected into the other ear as a control. After 24 hours the thickness of each ear was measured using a micrometer by an operator unaware of the treatment status of the individual animal. Results were expressed as the difference in thickness (mm) between the OVA and saline-treated ear (Δ ear thickness).

ii) In Vitro Lymphocyte Proliferation Assays

In vitro lymphocyte proliferation assays were performed essentially as described in section 5.3.2. Briefly, lymphoid cells were harvested from either the spleen or the popliteal lymph node draining the site of immunization, and single-cell suspensions prepared. Triplicate wells were seeded with 2x10⁵ cells in the presence or absence of 10µg/ml OVA for 48 hours, followed by an 18 hour incubation with ³H-thymidine at 1.0µCi/well. Proliferative responses were expressed as a stimulation index (SI) calculated as previously described (see section 5.3.2).

iii) Humoral Immunity to OVA

IgG, IgM and IgA isotype antibody production to OVA was analyzed by ELISA using 96-well microtitre plates sensitized overnight with 5µg/ml of OVA in ELISA coating buffer (see section 4.2.2) for the detection of IgM and IgG antibody isotypes, or a 10µg/ml solution for IgA (100µl/well). After washing 3 times with PBST, the plates were blocked for 1 hour at room temperature with 100µl of PBST, followed by incubation with 100µl of serum prepared at the time points indicated in Figure 6.1. Sera were diluted 1/2000 in PBST for detection of IgG, or 1/50 for IgM and IgA. After an incubation period of overnight at 4°C for IgA, or 2 hours at room
temperature for IgG and IgM, bound antibodies were detected using alkaline phosphatase-conjugated, goat anti-rat IgG (Nordic Immunology, Tilburg, The Netherlands) specific for each isotype (IgG, IgM or IgA) diluted 1/2000 in PBST. After 2 hours at room temperature, optical density (OD) was measured as described in section 4.2.2.

6.3 RESULTS

6.3.1 Cell-mediated Immunity

To assess the influence of oral feeding of OVA on the subsequent cell-mediated immune response to systemic OVA challenge, in vivo (DTH) and in vitro (lymphocyte proliferation) assays were performed 30 days after the final OVA feed using saline-fed Tx-X or normal animals as controls.

i) DTH Responses

The DTH responses of Tx-X and normal animals to OVA are shown in Figure 6.2. Despite the severe lymphopaenic state of Tx-X rats, these animals were still capable of mounting a DTH response to OVA equivalent to that of normal animals. In the case of Tx-X rats however, prior feeding of OVA was capable of suppressing the DTH response when compared with saline-treated Tx-X controls ($P<0.03$). Feeding of OVA to normal animals also suppressed the DTH response to OVA when compared to control animals, although the difference did not prove statistically significant.
Figure 6.2. DTH responses of OVA-fed and control, Tx-X and normal, PVG rats to OVA. OVA or saline-fed animals (n=5 for all groups) were established as described in Figure 6.1 and DTH responses assessed 30 days after feeding by injecting 20μg of OVA intradermally into one ear and an equivalent volume of saline into the other. After 24 hours the difference in thickness (Δ) between the OVA and saline treated ears was determined by micrometer.

* P <0.03 compared with saline-treated Tx-X rats (Student's t-test).
ii) *In Vitro* Proliferative Responses

Similar to DTH responses, the *in vitro* proliferative responses of Tx-X rat lymphocytes to OVA were also suppressed when compared to controls as a result of feeding the antigen prior to immunization (Figure 6.3). This tolerizing effect was seen for cells isolated from both the spleen and to a greater extent from the lymph node draining the site of immunization, however this did not reach statistical significance.

### 6.3.2 Humoral Immunity

To determine the ability to induce humoral tolerance to OVA in Tx-X rats, serum samples taken at various time points during the experiment were analysed for titres of IgG, IgM and IgA antibody isotypes to OVA (Figure 6.4). For IgG, both OVA-fed Tx-X (*P* <0.005) and normal rats (*P* <0.001) showed significantly suppressed antibody responses to OVA by day 29 compared with saline-fed controls. Interestingly, control Tx-X rats generated equivalent titres of IgG anti-OVA antibodies as saline-fed normal rats. This indicates that, at least for IgG production, Tx-X rats were capable of being orally tolerized to the same degree as normal animals. In addition, the ability of saline-fed Tx-X controls to mount a substantial IgG response to a T-dependent antigen such as OVA indicates that the observed suppression of IgG response to systemic OVA induced by prior OVA feeding was not simply due to the inability of Tx-X animals to mount a sufficient humoral response to the antigen.

IgM responses were similar for all groups except at day 9, when OVA-fed normal rats showed significantly raised IgM titres compared with other groups (*P* <0.01). This probably represented a primary IgM response
Figure 6.3. Proliferative responses to OVA of spleen and lymph node cells from Tx-X and normal PVG rats. Spleen and lymph node cells were isolated from OVA- or saline-fed Tx-X and normal rats 27 days after OVA immunization (n=5 or * n=3). After culturing for 48 hours in the presence of 10µg/ml OVA, cell proliferation was determined by ³H-thymidine incorporation.
Figure 6.4. Anti-OVA antibody responses of OVA-fed Tx-X and normal PVG rats. Titres of anti-OVA IgG, IgM and IgA antibody isotypes were determined by ELISA using serum samples taken at various time points after OVA feeding and measuring optical density at 405nm (OD\textsubscript{405nm}). Rats were immunized with 200µg of OVA on day 9 post-feeding (arrowed). $P < 0.005$, $\dagger 0.0001$ and $\ddagger 0.003$ compared with saline-fed controls. $§ P < 0.01$ compared with OVA-fed Tx-X value.
towards OVA encountered orally, as the bleed at this time point was taken prior to the OVA immunization. That these animals had begun to respond to oral OVA was also evident by the raised levels of IgM in the "time 0" bleeds, both in normal and Tx-X OVA-fed animals. By day 15, however, the IgM antibody titre of this group had dropped to levels similar to the other 3 groups.

For serum anti-OVA IgA, although low titres were observed, all groups showed increasing levels prior to systemic OVA challenge at day 9 (Figure 6.9). OVA-fed Tx-X animals did not then respond to systemic challenge and by day 29 showed significantly suppressed responses compared with all other groups ($P < 0.01$). OVA-fed normal PVG rats, however, showed enhanced IgA response after systemic challenge, perhaps indicating a priming effect. In contrast, saline-fed animals (both normal and Tx-X) tended to show steadily increasing in IgA levels throughout the experiment.

**6.4 DISCUSSION**

The above studies were undertaken to investigate the ability of Tx-X rats to tolerate antigens encountered via the oral route. As previous studies have shown, the induction of autoimmunity in Tx-X rats is able to be modulated as a result of manipulation of the gastrointestinal tract. Thus it was of particular interest to determine whether modulation of the immune system by Tx-X could lead to an enhanced positive (immunizing) or suppressive (tolerizing) immune response to intestinal antigens.
For cell-mediated immunity to orally-encountered OVA, four 100mg doses of OVA prior to parenteral OVA challenge were capable of inducing a state of immunological unresponsiveness in Tx-X rats. This was demonstrated *in vivo* as assessed by a reduced DTH response in OVA-fed Tx-X animals compared with saline treated controls (Figure 6.2) and also *in vitro* as assessed by lymphocyte proliferation to OVA (Figure 6.3). In the latter case, a greater degree of tolerance was seen with cells isolated from the lymph node draining the site of immunization when compared with cells from the spleen. This may have reflected the poor proliferative responses of spleen cells from Tx-X rats *in vitro* as a result of increased suppressive factors within these cultures (see section 5.4), or may have been due to an increased immunoregulatory function of cells from this site.

For humoral responses, oral administration of OVA was able to suppress IgG responses to systemic OVA in both Tx-X and normal animals by day 29 of the experiment (Figure 6.4). Of particular note in this case was the ability of saline-fed Tx-X rats to generate high titres of IgG antibody to OVA, indicating that although profoundly lymphopaenic, Tx-X rats were still capable of initiating strong humoral responses to T-dependent antigens. For IgM, no significant differences in antibody titre were observed except at day 9, where OVA-fed normal rats showed significantly raised IgM levels (Figure 6.4). As the serum samples at this point were taken before systemic OVA challenge, this probably represented a primary IgM response to orally-derived OVA, which was less evident in Tx-X animals. By day 29 however, no differences were observed in IgM titres between normal and Tx-X rats.

Being the primary regulatory immunoglobulin of the GI tract, the isotype of potentially the greatest interest was IgA. For serum IgA, a heterogeneous
response was observed to OVA between groups (Figure 6.4). Nevertheless, there was a tendency towards OVA-fed normal animals showing an enhanced, or primed, response to systemic OVA after feeding, while OVA-fed Tx-X animals showed significantly suppressed IgA levels by day 29 ($P < 0.01$). As seen for IgG, Tx-X animals were capable of generating an IgA response equivalent to that of normal animals, indicating that there was no deficiency in IgA production in Tx-X animals. From these results, however, it was evident that IgA was present in the serum at very low concentrations. Future studies of salivary or intestinal IgA levels, where antibody titres will be presumably increased, may further highlight the differences between treatment groups.

An isotype-specific antibody response has also been seen in other systems after feeding of antigen. For example, Fuller et al. have shown that oral administration of MBP to Lewis rats prior to MBP immunization suppressed serum IgG and IgA responses, while salivary IgA levels were increased (Fuller et al., 1990). These workers suggested that antigen-feeding influenced B-cell development by inhibiting T-cell derived lymphokines involved in B-cell maturation and immunoglobulin class switching, such as IL-4, 5 and 6. This hypothesis was supported by the recent studies of Hoyne et al., who demonstrated that OVA orally-tolerized mice show a modified lymphokine production, including undetectable levels of IL-4 (Hoyne et al., 1993).

Of interest in the present study was the inability to induce cell-mediated tolerance to OVA in normal PVG rats, as indicated by normal DTH and proliferative responses of OVA-fed when compared with saline-fed rats. A number of factors have been shown to the influence the induction of tolerance in rodents, including the age of the animal and the dose and
timing of the antigen feed. For example, in adult mice, a single dose of OVA is capable of inducing systemic tolerance to the antigen, both humoral and cell-mediated (Ferguson et al., 1988). In rats however, oral tolerance to OVA was only found to be inducible in young animals receiving much larger doses of OVA (Wold et al., 1988). In contrast, newborn mice have been shown to be less sensitive to the induction of oral tolerance (Strobel and Ferguson, 1984). Faria et al. also showed that strains of mice susceptible to the induction of oral tolerance at 8 weeks of age become refractory at 24 weeks of age (Faria et al., 1993). This same group also demonstrated that the outcome of feeding antigen, that is, tolerance or immunization, is affected by the dose and timing of feeding.

There have also been reports of intestinal microflora influencing the ability to induce oral tolerance, as germ-free mice remained tolerant to OVA after feeding for a shorter period than conventional animals, suggesting that the intestinal flora may act as regulatory factor in intestinal immune responses (Moreau and Corthier, 1988). In this respect, it would be of interest to compare the ability to orally-tolerize conventionally-derived and SPF-derived Tx-X rats, especially considering that the former are refractory to the induction of IDDM by Tx-X (see section 3.3.2).

In the present study, the age of the rats used, the dose of OVA administered and the time of feeding may all have influenced the observed outcome in relation to oral tolerance to OVA in both Tx-X and normal rats. This has been supported by a previous study where Tx-X rats were fed from 1 to 4 doses of OVA (100mg per dose) soon after final irradiation (P Deplazes, P Stumbles and J Penhale, unpublished observations). This was in contrast to the present study, where rats were fed 8 weeks after final irradiation. In this earlier study, Tx-X animals fed high doses of OVA
(4 feeds) exhibited both enhanced humoral and cell-mediated responses to OVA when compared to those receiving low doses, indicating an immunizing or priming effect and thus contrasting with the present study. In addition, 4 doses of OVA induced a tolerizing of cell-mediated immunity to OVA in age-matched normal control animals, but this was not observed for the humoral (IgG) response, and this also contrasts with the results of the present study.

In conclusion, this study has shown that Tx-X rats are capable of being orally tolerized to systemic antigen challenge, both at the cell-mediated level and in an isotype-specific manner at the humoral level (IgG but not IgM), by this particular feeding protocol. Tx-X rats appeared to be more susceptible to orally-induced tolerance to OVA than normal animals of the same age, suggesting an abnormality in the regulation of intestinal immunity in these rats. This was supported by a previous study in younger Tx-X rats that demonstrated a priming, rather than a tolerizing, response to systemic challenge with OVA after prior antigen-feeding. In both the present and this previous study however, it was possible to demonstrate that the immune response of Tx-X rats to antigens encountered via the oral route differed from that of normal animals and it is conceivable that a defect in tolerance to intestinally-derived antigens may influence the pathogenesis of autoimmunity in Tx-X animals. Further studies are required to establish this phenomenon, however, and to determine the role of altered intestinal immunity in the pathogenesis of Tx-X induced autoimmune disease.
These studies have described the clinical, histopathological and immunological features of a recently-identified diabetic syndrome induced in an inbred rat strain using a combination of thymectomy and irradiation (Tx-X), and have shown that the syndrome can consistently be induced in animals not normally prone to the spontaneous development of this disease.

The key clinical and histopathological features of the Tx-X syndrome, including the rapid onset of hyperglycaemia, the absolute dependency on insulin therapy and the presence of severe islet pathology in affected rats (Chapter 3.0), showed strong similarities to the IDDM syndrome of man. Furthermore, the Tx-X-induced syndrome was similar to the diabetic syndromes of both the BB rat and NOD mouse in many respects, further highlighting the value of this model for the study of diabetes initiation and pathogenesis (Table 7.1). Tx-X rats, however, differed from the human and NOD mouse conditions (but similar to BB rats) in that these rats had a severe lymphopaenia induced by the manipulation procedure.

The ability to induce disease by a non-specific manipulation of the immune system strongly suggests an immune-mediated origin for this syndrome. Furthermore, the ability to induce disease in an animal strain not normally prone to this disease implies that this procedure is in some way unmasking the autoreactive potential of a population of autoaggressive cells that are under regulation in the normal animal, a hypothesis previously postulated for thyroiditis induced by the same procedure (Penhale et al., 1973), but equally relevant to the pathogenesis of diabetes. Support for this effector/regulator imbalance hypothesis was provided in the present study by the ability to restore the immuno-
Table 7.1. Comparison of the Tx-X, human, BB rat and NOD mouse diabetic syndromes.

<table>
<thead>
<tr>
<th></th>
<th>Tx-X</th>
<th>Human</th>
<th>BB Rat</th>
<th>NOD Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of Onset</td>
<td>7-56 days after</td>
<td>Any age *</td>
<td>50 - 120 days</td>
<td>70 - 210 days</td>
</tr>
<tr>
<td></td>
<td>final X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incidence</td>
<td>35-40%</td>
<td>= 0.2%</td>
<td>80-90% †</td>
<td>70-80% †</td>
</tr>
<tr>
<td>Sex Susceptibility</td>
<td>Male&gt;Female</td>
<td>Male ≥ Female</td>
<td>Male &gt; Female</td>
<td>Female &gt;&gt; Male</td>
</tr>
<tr>
<td>Hyperglycaemia</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Insulinopaenia</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Ketoacidosis</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Insulitis</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Islet Atrophy</td>
<td>Detectable</td>
<td>Detectable</td>
<td>Detectable</td>
<td>Detectable</td>
</tr>
<tr>
<td>ICA</td>
<td>Detectable</td>
<td>Detectable</td>
<td>Not Detectable</td>
<td>Detectable</td>
</tr>
<tr>
<td>ICSA</td>
<td>?</td>
<td>Detectable</td>
<td>Detectable</td>
<td>Detectable</td>
</tr>
<tr>
<td>IAA</td>
<td>Detectable</td>
<td>Detectable</td>
<td>Detectable?</td>
<td>Detectable</td>
</tr>
<tr>
<td>64kDa Antibodies</td>
<td>Detectable</td>
<td>Detectable</td>
<td>Detectable</td>
<td>Detectable</td>
</tr>
<tr>
<td>Lymphopaenia</td>
<td>+++</td>
<td>±</td>
<td>+++</td>
<td>±</td>
</tr>
<tr>
<td>CD4⁺ T cells</td>
<td>Decreased ‡</td>
<td>Decreased</td>
<td>Decreased</td>
<td>Normal-Increased</td>
</tr>
<tr>
<td>CD8⁺ T cells</td>
<td>Decreased ‡</td>
<td>Increased</td>
<td>Decreased</td>
<td>Normal-Increased</td>
</tr>
<tr>
<td>CD4:CD8 ratio</td>
<td>Abnormal?</td>
<td>Abnormal?</td>
<td>Abnormal</td>
<td>Normal</td>
</tr>
<tr>
<td>Ia Expression</td>
<td>Increased</td>
<td>Increased</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>Anti-β cell CTL</td>
<td>?</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Thyroiditis</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
</tr>
</tbody>
</table>

* Most common before 20 years of age
† Varies according to subline or colony
‡ As determined by Fowell and Mason, 1993
regulatory capacity of Tx-X animals by transfusion of lymphocytes isolated from unmanipulated, syngeneic donors (Table 5.1).

Further support for this hypothesis has also recently been provided by the studies of Fowell and Mason, who have identified the regulatory cell of normal animals capable of preventing the expression of Tx-X-induced diabetes to be a CD4+ T cell of the phenotype CD45RClo TCRαβ+ RT6+ Thy-1- OX40- (Fowell and Mason, 1993). Cells of this phenotype have been shown to generate low levels of IFNγ but significant levels of IL-2 and mRNA for IL-4, IL-10 and TGFβ1 (Fowell et al., 1991 and D. Fawell, personal communication), thus suggesting them to be of a Th2-like phenotype. In addition, studies in the BB rat have also shown that injection of a CD4+ RT6+ T cell can prevent the onset of disease in this animal (Mordes et al., 1987).

Evidence for the activation of autoaggressive effector cells by the Tx-X procedure was provided in this study by the ability to transfer islet lesions to normal, unmanipulated syngeneic recipient rats using ConA-activated lymphoid cells isolated from diabetic donors (Table 5.2). Although this study was not designed to determine the characteristics of the autoreactive cells involved, several studies in other animal models have suggested that both CD4+ and CD8+ T cells are required. In the NOD mouse, in vitro depletion of either CD4+ or CD8+ T cells inhibits disease transfer (Bendelac et al., 1987; Christianson et al., 1993), while in vivo depletion of either of these cell types inhibits disease (Wang et al., 1987; Charlton et al., 1988). In the BB rat, CD4+ T cells alone appear capable of transferring the disease (Metroz-Dayer et al., 1990), however in vivo depletion of CD8+ T cells inhibits the disease onset (Like et al., 1986). Similarly, for the Tx-X model, in vivo depletion of CD8+ T cells completely
inhibited the disease, while transfusion of CD8+ T cells from normal donors restored disease susceptibility of CD8+ T cell-depleted Tx-X rats (Fowell and Mason, 1993).

That the β cell damage observed in Tx-X animals was cell-mediated was also supported by the detection of insulitis in a large proportion of Tx-X animals, whether diabetic or otherwise (Table 3.2). Although this lesion had given way to islet atrophy in acutely diabetic animals, a significant proportion of 'post-diabetic stage' Tx-X rats (that is, rats unlikely to ever to develop clinical disease) showed insulitis at necropsy. This indicates that a 'sub-clinical' form of the disease also occurred in some Tx-X animals, although how these animals managed to control this immune attack on the β cells and maintain normal carbohydrate metabolism remains to be determined.

Taken together, these data are supportive of the conclusion that islet β cells of Tx-X rats are destroyed by a cellular immune response, possibly mediated by a cytolytic CD8+ T-cell (Fowell and Mason, 1993). Furthermore, the ability to suppress the onset of disease in Tx-X animals by reconstitution with normal lymphocytes, particularly those of the CD4+, CD45RClow phenotype (Fowell et al., 1991), suggests that the β-cell cytolytic CD8+ T-cells are capable of being regulated by a sub-population of CD4+ T-cells which are being selectively depleted by the Tx-X procedure.

Of particular interest was the identification of increased numbers of activated macrophages in Tx-X rats (see section 5.4). The central role of macrophages in regulating the immune response, particularly with regard to antigen processing and presentation, is well documented. The recent
identification of nitric oxide (NO) as a potent immunoregulatory molecule, combined with the observation that NO synthesis is upregulated in activated macrophages (see section 5.4), provides a mechanism whereby activated macrophages could play a central role in the regulation/initiation of Tx-X-induced autoimmunity.

Considering all of the above observations regarding the regulation of cellular immunity and the interaction of T cell subsets, it is possible to propose a theoretical model for the interaction of activated macrophages and T cell subsets in the initiation and mediation of islet cell destruction in Tx-X rats (Figure 7.1). According to this model, the effector/regulator imbalance induced by Tx-X leads to, amongst other dysfunctions, increased macrophage activation. In addition, it is possible that the Tx-X procedure also alters tolerance to gastrointestinal antigens (addressed later in this chapter) and these may provide extra stimuli for macrophage activation. The consequences of this increased macrophage activity could be two-fold. First, in the microenvironment of the islet, NO synthesis and IL-1β production by resident activated macrophages leads to initiation of NO synthesis by β cells and thus their initial damage (Corbett et al., 1992). Antigens released by the damaged β cell are then processed and presented by both local and recruited antigen presenting cells, initiating and/or perpetuating a β-cell specific, cell mediated immune response. Secondly, on a more systemic level, the production of NO by activated macrophages in peripheral lymphoid tissue could favour the activation of a CD4+, Th1-type T cell by suppressing Th2-type CD4+ regulatory T cells, which in turn upregulates IFNγ production by Th1 CD4+ T cells, leading to further activation of macrophage NO synthesis (Albina et al., 1991). IFNγ itself would also act to suppress the activity of Th2 T cells (Fowell et al., 1991). Although NO acts as a non-specific inhibitor of cell function, a
Figure 7.1. Hypothetical role for activated macrophages in the induction and regulation of autoimmunity in Tx-X animals. See text for details.

NO - nitric oxide; Ag - antigen; CMI - cell mediated immunity.
differential effect of this molecule on Th1 and Th2 cells is suggested by the fact that NO production is enhanced by IFNγ (a Th1 cytokine), implying that cells producing this cytokine remain functional and are not suppressed by NO. In addition, IL-4 (a Th2 cytokine) is known to inhibit IFNγ-induced NO synthesis (Appelberg et al., 1992; Al-Ramadi et al., 1992), suggesting that cells producing this cytokine are likely to be suppressed in an environment of increased macrophage activation. Activated Th1 T-cells may then either participate in β cell destruction directly and/or provide cognate help for the activation of β-cell specific CD8+ cytotoxic T cells.

While this model is one of several that could be suggested, and represents an over-simplification of what may be happening in vivo, it does incorporate a number of in vitro and in vivo findings to date, both in the Tx-X and other models of IDDM. Further support for this model would be provided by in vivo macrophage inhibition studies, perhaps by silica treatment which interestingly has been shown to inhibit diabetes in both the BB rat (Oschilewski et al., 1985; Amano and Yoon, 1990) and NOD mouse (Charlton et al., 1988). Note also that this model supposes that rather than a single β-cell antigen, a number of antigens may act as targeting molecules for the β-cell specific autoimmune response, a situation that seems to be supported by the available experimental data (see section 2.4.2).

In addition to these cellular studies, further evidence for an autoimmune aetiology for diabetes induced by the Tx-X procedure was provided by humoral studies identifying autoantibodies to islet cell components by a number of techniques (Chapter 4.0). Generally however, these antibodies were present at low titres and were difficult to detect. Nevertheless, the
identification of ICA by immunofluorescence and antibodies to islet cell components and a 64kDa islet cell protein by immunoblotting and immunoprecipitation respectively add further support to the similarities between this model and the human syndrome. In addition, the concurrent development of autoantibodies to thyroid components, and the cellular infiltration of thyroid glands in Tx-X rats provides support for an autoimmune pathogenesis for diabetes and thyroiditis in these animals.

The difficulty in detecting islet-specific autoantibodies, both in the Tx-X and other experimental autoimmune diabetes syndromes, questions the significance of these autoantibodies in the disease pathogenesis, although this indirectly provides further support for the activity of a Th1-like cell mediated immune response in Tx-X animals, of which reduced help for antibody production is a characteristic feature (Fowell et al., 1991). Autoantibodies do, however, provide a tool for the identification of potential autoantigens, the value of which was highlighted by the difficulty in detecting T cell reactivity to islet cell and soluble proteins in Tx-X rats (section 5.3). Thus, the Tx-X model should prove useful for the identification of potential diabetic autoantigens and their subsequent in vivo analysis.

Although autoimmunity arising from an effector/regulator cell imbalance is a likely pathogenic mechanism for disease induction in Tx-X rats, it is clear from the data that other factors are involved in the aetiology of autoimmune disease in these animals. Thus, diabetes expression is a strain-related phenomenon, with PVG strain rats being particularly susceptible, but also with some indication that incidence may be influenced to an extent by the RT1u haplotype of the MHC (Table 3.4). This MHC haplotype is unquestionably linked to disease susceptibility in
the BB rat, and there is strong evidence to suggest that susceptibility to IDDM in both humans and NOD mice is also under strict genetic control (see Chapter 2.0).

The influence of gender on the expression of diabetes in Tx-X rats was less clear, although there was a tendency towards an increased disease expression in males (see Table 3.3 and Figure 3.10). A gender difference appears to be more pronounced in RT1<sup>u</sup> congenic rats, however, where Tx-X leads to a 100 percent incidence of diabetes in males and around 70 percent in females (Fawell et al., 1991). A similar situation exists for both humans and the BB rat, where males are marginally more susceptible (Table 7.1). Interestingly, in the NOD mouse females are significantly more susceptible, and a similar observation has been made for the expression of thyroiditis in Tx-X rats, where steroid hormones have been shown to have a profound influence on susceptibility to this disease (Ansar Ahmed and Penhale, 1982; Ansar Ahmed et al., 1983).

Of particular interest was the observation that the environmental status of the animal could influence the induction of disease by Tx-X. Thus, conventionally reared animals were significantly less susceptible to Tx-X-induced diabetes than their SPF-derived counterparts, both in terms of acute diabetes and islet lesions (see Table 3.3). It therefore appeared that a protective environmental agent, presumably of gastrointestinal origin, was present in conventional animals capable of suppressing the induction of diabetes. As none of the common rat viral pathogens could be detected in either SPF or conventional animals, it is reasonable to assume that this effect is due to differences (either qualitative or quantitative) in their intestinal bacterial flora. That intestinally-derived antigens are capable of modulating the expression of disease in Tx-X animals has
reconstitution of SPF animals with intestinal contents from conventional rats resulted in an enhanced disease expression (Penhale and Young, 1988). Thus, it is clear that environmental agents, probably gastrointestinally derived, are capable of modulating autoimmune disease induction in Tx-X rats.

One significant question raised by these observations is whether the process of thymectomy and irradiation alters the ability of such treated animals to remain immunologically tolerant to intestinal antigens. A loss or disruption of tolerance to orally presented antigens could provide a mechanism whereby intestinally-derived bacterial antigens could influence the expression of autoimmune disease in these animals. To test the hypothesis that Tx-X animals may have an abnormal tolerance to orally encountered antigen, Tx-X rats were fed a soluble protein antigen (OVA) and their immune response towards this antigen examined (Chapter 6.0). Although this set of experiments indicated that Tx-X rats were capable of being tolerized to OVA via the oral route, these animals did behave differently to the age-matched, non-Tx-X control rats. Nevertheless, it is also very likely that several factors could influence the outcome of this type of experiment, such as the age of the animal, the dose and frequency of antigen feeding, supported by previous studies in younger rats where a priming or a tolerizing affect could be observed in Tx-X animals depending on the dose of antigen fed (see Chapter 6.0).

In light of the ability of intestinal factors to modulate disease expression in Tx-X animals, and the possible disruption of the ability of these animals to tolerate orally-derived antigens, it is possible to speculate on a mechanism whereby increased leakage of potentially autostimulatory factors from the gut, coupled with deregulated immunoregulation towards these factors,
may be operating in Tx-X animals to provide a trigger for the initiation of an autoimmune response. A role for environmental agents in the aetiology of a wide range of autoimmune diseases has long been postulated, but to date still remains controversial. A mechanism often proposed for the ability of environmental, and in particular microbial (bacterial or viral), agents to trigger an autoimmune response is that of molecular mimicry, a process whereby an epitope of a microbial antigen mimics that of a tissue autoantigen, thus leading to the possibility of a cross-reactive immune response.

Molecular mimicry has been suggested as a possible mechanism contributing to the aetiology of a number of autoimmune diseases, although direct evidence for this phenomenon has been difficult to establish (Oldstone, 1989). Nevertheless, there is evidence from other models of autoimmune disease, particularly those of arthritis, where environmental conditions can alter the course of the disease, while in humans, molecular mimicry of antigens of several bacterial strains probably leads to the induction of arthritis in susceptible individuals (van Eden, 1991). For diabetes, insulin or a related molecule has been described in strains of *E. coli* (Le Roith et al., 1981), and a potential β cell autoantigen glutamic acid decarboxylase (GAD - see Chapter 2.0) has long been known to be an active bacterial enzyme (Strausbauch and Fischer, 1970). In support of this, GAD-producing strains of bacteria have recently been isolated from the gut of diabetic Tx-X rats (J Koppen, personal communication). Similarly, intestinal bacteria are known to have peroxidase activity, thus presenting the possibility for cross-reactivity with the putative autoantigen of thyroiditis (thyroid peroxidase). Thus, there is enormous potential for immunological cross reaction between gut-derived bacterial proteins and tissue antigens, although the extent to which
molecular mimicry can influence the course of autoimmunity in Tx-X rats remains to be determined.

In conclusion, this study has shown that a diabetic syndrome closely resembling human IDDM of man can be induced in rats not normally prone to this disease by a combination of thymectomy and irradiation (Tx-X). The evidence presented in this study strongly supports the conclusion that the specific destruction of $\beta$ cells observed in this syndrome is of autoimmune origin, resulting from a regulator/effecter cell imbalance in these animals that activates a population of latent autoaggressive cells that in the normal animal are under strict control. How these cells escape early deletion in the thymus remains an intriguing question. The fact that other strains examined in this study were not nearly so susceptible to diabetes induction suggests that PVG rats may have a primary defect in this deletion process.

In addition, evidence has been provided demonstrating that genetic and environmental factors are both capable of influencing the aetiology of Tx-X-induced autoimmunity, a situation mirroring that of the human disease. The suitability of this model for the study of IDDM pathogenesis was further highlighted by the ability to manipulate the Tx-X syndrome towards inducement or inhibition, while the lack of the genetic constraints observed in other animal models of IDDM should provide additional opportunities for the study of the role of cell mediated immune mechanisms in this disease. Finally, the close similarities between this syndrome and the clinical disease of humans provides further evidence for an immune-mediated pathogenesis for IDDM.
CHAPTER 8.0
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