IMMUNOLOGICAL STUDIES IN MULTIPLE LOW DOSE STREPTOZOTOCIN INDUCED DIABETES IN MICE

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Immunological Studies in Multiple Low dose Streptozotocin Induced Diabetes in Mice

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SUMMARY

1. Multiple low doses of streptozotocin (MSZ) treatment successfully induced diabetes in male TO, MF1 and HO lean mice. In contrast however, BALB/c mice failed to develop persistent hyperglycaemia. Single streptozotocin (SSZ) treatment also produced diabetes in TO mice. SSZ treatment however, produced severe weight loss and atrophy of the lymphoid organs. MSZ treatment on the other hand, was not cytotoxic towards lymphoid organs and, whilst there was no loss of body weight, growth rates were reduced in MSZ treated mice.

2. Following sheep red blood cell (SRBC) immunisation of MSZ-treated mice, haemagglutination titres, and numbers of antigen reactive cells and plaque forming cells were all significantly lower than control values.

3. In-vitro proliferation of spleen cells in response to phytohaemagglutinin (PHA) and conconavalin A (ConA) was found to be significantly depressed in MSZ treated mice. However, T-lymphocyte responses were intact when the mice were not overtly hyperglycaemic. In contrast, however, T cell independent responses to lipopolysaccharide (LPS) were generally intact throughout the study period.

4. Cell mediated immunity, as assessed by measurements of delayed (Type IV) hypersensitivity, was also depressed in MSZ treated mice. This suppression could be reversed by insulin therapy.

5. Both natural killer cell activity and antibody dependent cell mediated cytotoxicity were found to be significantly increased in MSZ treated mice.

6. Histological examination of the pancreas showed the presence of insulitis, in MSZ treated mice, and cytotoxic effector cells against obese mice islet cells (as assessed by ^{51}Cr release) and HIT-T15 cells (as assessed by insulin secretion) were found to be significantly increased. Furthermore, these effector cells were also found to show increased proliferation in the presence of homogenates prepared from HIT-T15 cells. Examination of the sera from MSZ treated mice showed that islet cell surface antibodies were present.

Key Words: Multiple low dose streptozotocin, diabetes, immune responses, anti-β-cell mediated immunity.

In the name of Allah the Almighty, Most Merciful and Compassionate.

I dedicate this thesis to the memory of my mother and also to my father and brothers.

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CHAPTER 1. GENERAL INTRODUCTION

1.1 DIABETES MELLITUS

Insulin plays a central role in the metabolism of carbohydrate and fat, it regulates the utilisation and storage of both these substances. In the 'fed' state, insulin facilitates the uptake of glucose by insulin sensitive tissues such as liver, muscle and adipose tissue, and brings about its storage as glycogen in the liver and muscle, or as fat in the adipose tissue.

Diabetes mellitus results from an absolute or a relative lack of insulin. This may be brought about by a number of mechanisms including insufficient secretion of insulin by the β cells of the pancreatic islets, or the presence of high titres of antibodies to insulin receptors on the peripheral tissues (i.e. insulin resistance) which block or inhibit insulin binding (Kahn 1984). Insufficient insulin secretion may arise as a result of a number of factors, e.g. death or impaired function of islets or β cells caused by autoimmune, genetic, chemical or viral mechanisms (Cahill and McDevitt 1981).

When insulin is absent or much reduced, there are major defects in the storage and utilisation of carbohydrates and fats. Thus, in the fed state, glucose is not removed from the blood by insulin sensitive tissues and hyperglycaemia results. Excessive amounts of glucose in the blood may exceed the renal threshold for its reabsorption and glucose appears in the urine (glycosuria) leading to excessive loss of water (polyuria). In an attempt to correct what appears to the body to be a lack of blood glucose, glycogen, fat and proteins are broken down. Glycogen is broken into

glucose; triglycerides (fat) are broken into glycerol and free fatty acids; and proteins are broken down to amino acids. The free acids are converted by the liver into β-hydroxybutyric acid and acetoacetic acid (ketone bodies). Whilst the glycerol is converted into glucose by gluconeogenesis. The body utilises glucose, ketone bodies and free fatty acids for energy metabolism. However, the gluconeogenesis and glycogenolysis processes lead to further hyperglycaemia and glucose excretion. The breakdown of the protein and fat contributes to weight loss and an excessive accumulation of ketone bodies (ketosis). Ketosis leads to increased blood acidity, whilst excretion of ketone bodies results in the loss of Na^+ and K^+ ions in the urine. Ultimately, diabetic coma and death may ensue. More detailed accounts of carbohydrate metabolism can be found elsewhere (Taylor and Agius 1988).

The above events, experienced by diabetic individuals are a result of a severe and acute shortage of insulin, when little or no attempt is made to alleviate the situation. Usually however, insulin therapy and/or dietary control can alleviate or prevent these problems. Nevertheless, even with these attempts to regulate carbohydrate metabolism, efficient metabolic control is not always attained and several disorders may arise as a consequence. Thus, for example, both hyperglycaemia and high levels of free fatty acids have been reported to depress immune responses in-vitro (Mertin et al 1974; Selam et al 1979).

1.2 CLASSIFICATION OF DIABETES

Current evidence suggests that diabetes is a heterogeneous group of disorders. It is important, therefore, to be able to distinguish adequately between the different forms, so that appropriate treatment can be administered. In the past; diabetes was divided into primary or idiopathic diabetes mellitus, and also into those forms in which diabetes was secondary to certain other conditions and syndromes. (Table 1.1).

It became apparent from clinical studies that primary diabetes could be separated into two distinct types classified according to the age of onset. These are Juvenile onset diabetes (JOD) and Maturity onset diabetes (MOD), the age of division being arbitrarily set at around 25-30 years. Later, this classification was considered unsatisfactory due to the great overlap in age of onset and symptoms between the two types, and many different classifications were proposed to satisfy the growing confusion in the literature (Bottazzo and Doniach 1976; Irvine 1977; Cudworth et al 1981).

In 1979, the National Diabetes Data Group (NDDG), in conjunction with many international associations for diabetes, proposed a new classification for diabetes mellitus (NDDG, 1979). This classification is based, not on age, but according to the therapy required. These are Type I diabetes, otherwise termed insulin dependent diabetes mellitus (IDDM), and Type II diabetes, also called non-insulin dependent diabetes mellitus (NIDDM).

Table 1.1 Classification of diabetes mellitus. Simplified from the classification scheme prepared by the National Diabetes Data Group (1979)

	Class	Associated Factors	Clinical Characteristics
Primary diabetes	Insulin dependent diabetes mellitus (IDDM) Type I	Evidence regarding aetiology suggests genetic and environmental factors, association with certain HLA types and autoimmune reactions	Insulin dependent. Characterised by insulinopenia. Islet cell antibodies are frequently present at diagnosis
	Non-insulin dependent diabetes mellitus (NIDDM) Type II (1) Non-obese NIDDM (2) Obese NIDDM	Obesity is suspected as an aetiological factor. Environmental and genetic factors are probably involved	Non-insulin dependent. Hyperinsulinaemia and insulin resistance characterise some patients in this sub-type
Secondary diabetes	Other types including diabetes mellitus associated with certain conditions and syndromes: (1) Pancreatic disease (2) Hormonal (3) Drug or chemical induced (4) Insulin receptor abnormality (5) Certain genetic syndromes (6) Miscellaneous	Aetiology can be known (e.g. diabetes secondary to pancreatic disease, endocrine disease or administration of drugs). In others aetiology is suspected because of higher frequency of association of diabetes with a syndrome or condition	In addition to the presence of specific conditions or syndromes, diabetes mellitus is also present

Type I diabetics are ketosis-prone individuals, who are dependent on daily injections of insulin for proper maintenance of metabolic control. Formerly referred to as JOD, this is the commonest form of diabetes, affecting individuals under the age of 30 years, but it is not infrequent in elderly people. Type II diabetics have a milder syndrome of hyperglycaemia and can be managed by regulation of carbohydrate intake, or by oral hypoglycaemic agents (OHA). This form was previously referred to as MOD; the patients are not prone to ketosis and are usually middle-aged or elderly. Furthermore Type II diabetes often affects those individuals who are obese. The criteria of insulin dependence, however, also seems inadequate because many NIDDM patients are treated with insulin, and some Type II patients develop clear insulin dependence. The overall prevalence of Type II diatetes exceeds Type I diabetes by about 10 : 1 (National diabetes data group, 1985) but due to the life threatening nature, it is the latter which has received the most attention from researchers.

The NDDG therefore, also proposed a parallel classification for the aid of the researcher (NDDG, 1979). The criteria for this sub-grouping are based on detection of islet cell antibodies (ICA); an association with organ-specific disorders; and HLA typing (Table 1.1). However, some caution needs to be exercised in the interpretation of this classification because it is based on studies carried out on diabetics in Europe and North America. The possibility exists that amongst other populations the characteristics of these types may be somewhat different. Indeed, there is evidence to suggest that there is marked ethnic variability in the prevalence and clinical features of diabetes mellitus (Cudworth and Wolf 1984). In any event, irrespective of the criteria chosen for subdivision in the classification, there are a small percentage of diabetic individuals who do not fit neatly into any group. The majority of diabetic patients

however, can be divided into the various subgroups and therefore the appropriate therapy can be applied.

1.3 CONCEPT OF AUTOIMMUNITY AND AUTOIMMUNE DISEASES

An autoimmune disease is a condition in which self components (i.e. the components of the body's own constituents) are recognised as antigens (autoantigens). When this happens, autoantibodies or cell mediated immune (CMI) reactions are induced against self components, which result in deleterious consequences. Under normal conditions the immune system is regulated and does not react aggressively against the body's own constituents. However, it is inevitable that sometimes, regulating mechanisms can go wrong and give rise to autoimmunity.

There is a large list of autoimmune diseases in man in which autoantibodies and CMI are present. These disorders tend to distribute themselves across a broad spectrum. At one end are the organ specific diseases, in which both humoral and cell mediated autoimmune reactions are confined to a particular tissue. Autoimmune thyroiditis (Hashimoto's disease) is one of the best examples of a strictly organ specific autoimmunity where the immune responses are usually confined to the thyroid gland. Serum antibodies have been found in at least three thyroid autoantigens, namely thyroglobulin, a microsomal fraction and a soluble colloid fraction distinct from thyroglobulin. The thyroid gland in autoimmune thyroiditis is also infiltrated by large numbers of mononuclear cells i.e. monocytes and lymphocytes (Roitt et al, 1985). At the other end of the spectrum are the non-organ specific diseases where autoimmune reactions are not confined to a single organ. This type of disorder is exemplified by systemic lupus erythematosis (SLE). The serum contains antibodies capable of specific interaction with various subcellular constituents found in most of the cells of the body. The most characteristic of these antibodies are directed at components of cell

nuclei (Holborn 1970). Pathological changes are widespread and are concerned primarily with lesions of connective tissue, affecting especially the kidney. Autoimmune disorders in the mid- spectrum are those disorders where the lesions tend to be confined to a single organ but the antibodies are non-organ specific. For example, in primary biliary cirrhosis, cell mediated immunity is directed mainly against the small bile ductules, but the serum contains autoantibodies not specific for the liver (for general review see Roitt et al 1985).

It is assumed that the presence of autoantibodies in the serum of individuals suffering from any of the autoimmune disorders are involved in tissue destruction. However, it is important to appreciate that such autoimmune reactions may themselves result as a consequence of the disease process (tissue damage) or some other factors (e.g. viral infections, see section 1.5) rather than being the primary cause of tissue damage. For example, heart autoantibodies are produced following myocardial infarction and, in this case, autoantibodies play no primary pathogenic role and are produced as a result of a secondary lesion. It might be that autoantibodies may play a non-aggressive role, acting as opsonins (coating of antigen with antibody, which facilitates the phagocytosis of the antigen) for autoantigens released from damaged tissues, thereby aiding their disposal. However, whilst autoantibodies may have a biologically useful function, we are concerned here with whether autoimmune reactions play a primary pathogenic role in the development of autoimmune disease.

In many autoimmune disorders the role of autoimmunity is thought to be the primary pathogenic factor. Even so, in the light of the present state of knowledge, it is

desirable to apply certain criteria that allow a disease to be considered as being of autoimmune aetiology. These criteria, summarised in Table 1.2, are presented as a general guideline, and are certainly relevant to disorders in which autoimmune aetiology has been well established (e.g. Hashimoto's disease). The same criteria can therefore be applied to investigate whether any disease may also have an autoimmune aetiology.

Table 1.2

Criteria for a disease to be considered as having autoimmune aetiology
(modified from MacLaren 1981)

1) Presence of circulating autoantibodies in the serum, these can be organ-specific or non-organ specific (humoral autoimmunity)

2) Presence of circulating lymphocytes sensitised to autoantigens (cell mediated immunity)

3) Identification of autoantigen(s)

4) Presence of infiltrating lymphocytes or plasma cells in the affected organ or tissue.

5) Passive transfer of autoimmune disease into experimental animals.

6) Prevention of the disease by immunosuppression.

1.4 AUTOIMMUNITY IN DIABETES

Much of the research examining the role of autoimmunity in diabetes mellitus stems from studies of well established autoimmune diseases, particularly organ specific endocrine disorders (e.g. Hashimoto's disease and Addison's disease). Thus, when autoantibodies or infiltrating lymphoid cells were found in the thyroid or adrenal glands, these were then looked for in islets of Langerhans from diabetic patients. Evidence for the involvement of autoimmunity in diabetes mellitus came from several studies; these will be discussed below.

In line with the criteria for the involvement of autoimmunity in any disease, it is appropriate at this stage to set the specific criteria for the involvement of autoimmunity in diabetes mellitus (MacLaren 1981). These criteria are presented in Table 1.3, however, it will become clear in due course that since diabetes is a heterogeneous disorder where autoimmunity is one of many possible aetiological factors; the criteria are only meant to serve as a guideline.

Table 1.3 Criteria for autoimmune aetiogenesis of diabetes mellitus.

(modified from Maclaren 1981)

1. Correlation with autoimmune endocrine diseases

2. Presence of serum autoantibodies to non-pancreatic tissue

3. Presence of specific autoantibodies directed against the β-cell

4. Mononuclear infiltration of islets of Langerhans

5. Presence of circulating sensitised lymphocytes to antigens on the β-cell

6. Induction of diabetes by an autoimmune method in experimental animals.

7. Passive transfer of diabetes e.g. transfer of T-cells from diabetic individuals into normal mice.

8. Prevention of diabetes by immunosuppression

Association of diabetes with other endocrinopathies

The earliest evidence that an immune mechanism may be involved in diabetes came from the observed association between IDDM and other endocrine disorders that were known to have an autoimmune aetiology. Although the co-existence of diabetes and Addison's disease (primary adrenocortical failure) for example has long been recorded, it was not until the observation that Addison's disease may have a significant autoimmune pathogenesis (Irvine and Barnes, 1975) that it was implied that diabetes may also have an autoimmune pathogenesis. Since then, numerous studies have indicated that IDDM occurs more frequently (30-50 times) in patients suffering from Addison's disease, than can be accounted for by chance alone (e.g. Nerup 1974). Conversely, Addison's disease has been found to occur 4 to 5 times more frequently in diabetics than in the general population (Kozak 1971; Nerup 1974).

Numerous reports have indicated an association between diabetes and impaired thyroid function (e.g. Landing et al 1963; Solomon et al 1965). These disorders of the thyroid constitute a number of heterogeneous autoimmune diseases including Hashimoto's thyroiditis, primary myxoedema and thyrotoxicosis. Furthermore, it has been found that Type I diabetes is closely associated with a number of other endocrine disorders, such as pernicious anaemia and autoimmune ovarian failure, which are known to have an autoimmune pathogenesis (Irvine 1980). Moulias et al (1974) reported that the endocrinopathesis mentioned above, are all intimately inter-related at the clinical level, i.e. a patient may have a number of disorders at the same time. Since Type I diabetes is also one disorder associated with the other well established

autoimmune disorders, it is suggested that autoimmune pathogenesis may also be important in Type I diabetes.

Autoantibodies to islet cells

The association between IDDM and other recognised autoimmune endocrinopathies provided circumstantial evidence for the involvement of autoimmunity in the aetiology of IDDM. Direct evidence for the latter was provided in 1974 when Bottazzo et al first described the presence of islet cell autoantibodies (ICA) in insulin dependent diabetics with coexistent autoimmune polyendocrine disease. The presence of ICA in diabetic patients was quickly confirmed by other workers (MacCuish et al 1974a). Subsequently, more detailed studies revealed ICA positive titres in 60 -70% of newly diagnosed IDDM patients, whilst ICA were observed in only 0.1 - 1% of the normal population (Lendrum et al 1975b; Irvine 1977; Bottazzo et al 1978).

The high prevalence of ICA in IDDM patients suggested that the autoantibodies may have a significant role in the development of diabetes. However, ICA were also found in the normal population, albeit only in a small percentage, which raised the question as to the purpose of ICA in these individuals. Nonetheless, the presence of ICA in non-diabetic patients can be explained by conferring a predictive role for ICA in the development of IDDM. In this case, ICA should be present in a small number of 'apparently normal' (prediabetic) individuals. It would follow that these prediabetic patients would subsequently progress to develop overt clinical diabetes. The presence of autoantibodies prior to clinical onset is a common feature of all organ specific autoimmune disorders (e.g. thyroid autoimmune disease and Addison's disease).

Therefore, studies were undertaken to determine the predictive nature of ICA. Autoantibodies were found in patients receiving OHA therapy, who later went on to develop IDDM (Irvine et al 1978). It was determined that the likelihood of ICA positive patients progressing to IDDM could be ranked in the following order, i.e. OHA treated patients > patients with impaired glucose tolerance > patients with normal glucose tolerance. (Irvine 1980). These results demonstrated that ICA were present in normal patients who had the least likelihood of developing IDDM. Furthermore, ICA were also found at a greater prevalence in patients who had impaired glucose tolerance or were treated with OHA; consequently these patients had a greater likelihood of developing IDDM compared to normal subjects. Patients who have impaired glucose tolerance or are treated with OHA can be considered as having a milder, or earlier stage of overt insulin dependent diabetes mellitus, because these patients often progress to develop IDDM. Since ICA are present in both control subjects and those in early stages of IDDM, it has been suggested that the presence of ICA may be of predictive value (Irvine 1980). However, the prevalence of ICA in IDDM falls with time and disappears within a year of detection, whilst in some patients with co-existing endocrinopathies it persists for much longer periods (Irvine et al 1977). On the other hand there are patients who do not have ICA at diagnosis of IDDM and subsequently do not go on to develop any antibodies (Lendrum et al 1978). Botazzo et al (1978) also found that 15-35% of IDDM patients did not develop any detectable ICA.

These variations may result from a heterogeneity in IDDM induction. Support for this comes from the detection of ICA following viral infection in normal patients who later

developed IDDM (Champsaur et al 1982). Furthermore, ICA have been found in first-degree relatives of diabetics who also developed IDDM (Srikanta et al 1983; Gorsuch et al 1981). These studies suggest that ICA can be induced by several mechanisms including autoimmune, viral and genetic factors.

Despite the presence of ICA in IDDM patients and its known predictive value for the onset of IDDM, their precise role (if any) in the aetiology of IDDM is not clearly understood. ICA have been found to react with all the cell species present in the islet (Bottazzo and Doniach 1978). This would imply that because of this non-specific binding ability, the pathological significance of ICA may be questioned. However, although ICA do bind with all cell species present in the islet, they do not alter the secretory capacity of non β-cells. Thus for example, glucagon and somatostatin secretion by α and D cells present in the islet is not decreased.

Lernmark et al (1978b) demonstrated another category of antibody in IDDM patients that could be detected by its ability to fix complement, such antibodies are called complement-fixing iselt-cell antibodies (CF-ICA). In newly diagnosed IDDM patients CF-ICA were found in 50% of patients that were ICA positive (Bottazzo et al 1980). Furthermore, it was determined that CF-ICA were predominantly detected in the sera with high-titre ICA (Bruining et al 1984). Thus suggesting that CF-ICA merely represent high titre ICA measured in a less sensitive assay, rather than a separate antibody class as previously reported. High titre ICA or CF-ICA have been reported to be of a significant marker for predicting the onset of IDDM (Betterle et al 1987).

Lernmark et al (1978a) demonstrated the presence of islet cell surface antibodies

(ICSA) in the sera of IDDM patients. These antibodies were specific for the cell membrane of β-cells and did not react with other cell species present in the islet (Van de Winkel et al, 1982). A pathogenic role for ICSA has been suggested since these antibodies are specific for β-cells (Brogren and Lernmark 1982). Furthermore, the identity of the antigen on the surface of the β-cell has been partially characterised (Baekkeskov et al 1982). The authors demonstrated that sera from recently diagnosed IDDM patients, positive for ICSA, recognised a protein of MW 64 KD in the surface of β cells, however recent studies suggest that the 64 KD protein lies in the cytoplasm (Bottazzo et al 1987). A recent report found that 95% of newly diagnosed IDDM patients had antibodies to a 64 KD islet cell protein,and that these autoantibodies preceded the onset of diabetes (Baekkeskov et al 1987).This report suggests therefore that the 64 KD islet cell protein is good marker for predicting IDDM.

Insulitis

In IDDM the ability of the islets to secrete insulin is considerably diminished. Furthermore, insulin content as well as the total islet volume is reduced and if the syndrome persists for 10-15 years, only a few patients retain some β-cell function (Gepts and De Mey 1978; Binder and Faber 1978). Although the exact mechanisms for the destruction of islets still eludes researchers, considerable evidence now exists for a cell mediated abnormality playing a significant role in the development of IDDM.

Evidence for the involvement of cell mediated immunity in IDDM comes from the observed presence of mononuclear cells (lymphocytes and monocytes) in islets of Langerhans of ketotic diabetics who have succumbed to the disease. Von Meyenburg

(1940) called this phenomenon insulitis. However, it was not until Gepts (1965) had realised that insulitis was frequently present in Type I diabetics that a general interest arose amongst researchers. The renewed interest in insulitis provided important insights into the histopathology of the diabetic pancreas. Affected islets lose the normal framework of the densely packed cell structure and show signs of pseudoatrophy. Immunohistochemical staining of these cells indicate that very few β-cells are present in such islets (Rahier et al 1983). When β-cells have been destroyed, lymphocytes progressively disappeared from the islets and lymphocytic infiltration was not found in islets devoid of β-cells. One interpretation of these findings is that the insulitis process is destroying β-cells whilst α, D and PP cells are not affected, except for an indirect effect of the loss of normal cell-cell interactions which may lead to small decreases in non-insulin producing cells (Foulis and Stewart 1984). Cell mediated immunity specific for β-cells is further implicated as a result of observations showing that insulitis reappears in atrophic islets where regeneration is occurring and destroys the newly formed cells (Gepts and Le Compte 1981).

Antipancreatic autoimmunity

After the observation that insulitis was found amongst insulin dependent diabetics (Gepts 1965), Nerup and his associates demonstrated that sensitised lymphocytes may be responsible for β-cell destruction in diabetic patients (Nerup et al 1971). The authors showed that leucocytes from IDDM patients did not migrate as much as the leucocytes from age matched control donors in a Leucocyte Migration Inhibition (LMI) assay, when exposed to a protein fraction prepared from duct ligated pancreas. Although the leucocytes used had not been purified, it was implied that the migration

inhibition may be due to the release of lymphokines from sensitised T-lymphocytes. This view was supported by an in-vitro study which showed that porcine pancreatic homogenate also evoked a delayed hypersensitivity reaction in IDDM patients (Nerup et al 1971).

The LMI test has been used to show that leucocytes from a mixture of IDDM and NIDDM patients gave a positive, but lower, migration inhibition against antigen prepared from an homogenate of foetal calf pancreas (Nerup et al 1974), and human pancreas (MacCuish et al 1974a). These values of migration inhibition from the above authors were lower when compared to that value observed by Nerup et al (1971), when only leucocytes from IDDM were tested. Thus implicating that positive LMI may be confined to IDDM patients rather than NIDDM diabetics. This observation was supported when a much higher migration inhibition was obtained using leucocytes from IDDM and using an extract of human insulinoma; a much purer antigen source (Irvine et al 1976a).

Interestingly, LMI reactivity did not correlate well with the possession of islet cell antibodies (Christy et al 1976b). One interpretation of these findings is that several different autoimmune mechanisms may be involved in the aetiology of IDDM.

Passive transfer

In-vivo studies of the interaction between specifically sensitized lymphocytes from IDDM and the intact cells of islets were made possible by the use of the athymic nude mouse in which T-cell-dependent immune mechanisms are severely compromised. Theoretically, the transfer of lymphocytes from diabetic patients which are positive for

the LMI test would cause insulitis and impaired glucose tolerance or diabetes in the recipients.This experimental approach however has produced controversial results.

Buschard et al (1978) transplanted peripheral lymphocytes from each of six patients with newly diagnosed IDDM into the peritoneum of athymic nude mice. During the 30-day follow-up study, blood glucose concentrations in some of the mice in the experimental group were significantly higher when compared with control mice which received lymphocytes from non-diabetic subjects. In contrast however, other reports have not been able to confirm this study (Lipsick et al 1979; Neufield et al 1978; Serra et al 1979).

Immunosuppression

Successful immunosuppression would be the final confirmation that immunological mediated processes are involved in B-cell destruction. Cyclosporin A, an immunosuppressive drug, has been reported to prevent diabetes in the spontaneously diabetic BB rat (Laupacis et al 1983). The same group later found that cyclosporin A treatment in insulin dependent diabetics was found to be beneficial (Stiller et al 1984). This observation has led to further and more detailed studies on the effect of cyclosporin A on the IDDM. Generally, the studies suggest that it can increase the rate of remission. Increased remission from IDDM diabetes has also been reported by therapy with other immunosuppressive drugs such as azathioprene and prednisone. Such studies may indicate that immunosuppression could be a possible tool for the treatment of newly diagnosed disease where only partial destruction of the β cells has occurred. However, much research is still needed to determine the value of

immunosuppression drugs, especially since cyclosporin A has been reported to be cytotoxic to the kidney and furthermore, withdrawal of the drug may precipitate the disease. Further details of these studies can be found elsewhere (e.g. Bottazzo et al 1987).

1.5 OTHER MECHANISMS IN THE AETIOLOGY OF IDDM

Considerable variations in the prevalence of diabetes have been documented in different countries (Report of WHO study group 1985). Genetic differences may account for some of these observed differences. However, a considerable variation in the prevalence of diabetes amongst populations with similar genetic backgrounds has also been reported (Zimmet, 1979). Polynesians and Micronesians undergoing modernisation of lifestyle exhibit a high prevalence of diabetes, whereas in their traditional culture the rates are low (Zimmet et al 1981).Similarly children born in New Zealand from Western Somoan emigrants showed a high prevalence of diabetes over children born in their place of origin (Elliot 1984) The exact reasons for these findings are unclear. Whilst many factors are likely to be involved, there is some evidence to suggest the changes in diet, especially from traditionally low protein to a high protein intake, may be important.

Circumstantial evidence suggests that ethnic groups who traditionally introduce cows milk into the diet of infants at an early stage have a high rate of childhood diabetes. Whereas those groups who suckle their infants for relatively longer periods of time, and do not have ready access to cows milk, have a low rate. This speculation is supported to some extent by a report which found that childhood diabetic patients were generally fed for a shorter period of time on human milk than their non-diabetic siblings or peers (Borch-Johnsen et al 1984). Studies on the spontaneously diabetic BB rat and NOD mouse have supported some of the above observations. A protein-free diet introduced before weaning to the diabetic prone BB rat reduced the onset of

diabetes (Elliot and Martin 1984; Scott et al 1985). A non-protein diet has also been reported to largely prevent diabetes in genetically susceptible NOD mice (Elliot et al 1988). It is possible therefore that variations in diet may have an important role to play in triggering IDDM in genetically susceptible individuals.

HLA Association with IDDM

By analogy with other endocrinopathies, a genetic component has also been proposed in the aetiology of IDDM. Certain autoimmune disorders are associated with specific antigens of the major histocompatibility complex (MHC) which constitute the human leucocyte system A (HLA). The HLA system (HLA-A, -B, -C, -D and -DR) is located on the short arm of the sixth chromosome, where genes that control many aspects of the immune response are located. HLA-A, -B and -C antigens occur as cell membrane glycoproteins present on all nucleated cells, whereas the HLA-D and -DR antigens are only present on some cells (e.g. B-lymphocytes, monocytes, endothelial cells). HLA studies have indicated that HLA-DR 3/4 antigens are found more frequently in individuals with IDDM, than in the normal population (Platz et al 1981, Wolf et al 1983), whilst HLA-DR2 antigens occur less frequently (Nerup et al 1984).

A possibility exists that there may be genetic heterogeneity within typical diabetes because more than one gene in the HLA complex is conferring susceptibility. Immunological studies in Type I diabetics with HLA-DR3, have revealed persistent islet cell antibodies (ICA). Furthermore, these patients had an increased incidence of association with other autoimmune disorders. In contrast, diabetics possessing DR4 antigens have transient ICA and their diabetes is not associated with other autoimmune

endocrine disorders (Irvine et al 1978). Genetic heterogeneity within Type I diabetes prompted researchers to propose that Type I diabetes could be subdivided into autoimmune (Type IB) and virally-induced (Type IA) types (Bottazzo and Doniach 1976; Irvine 1977; Rotter and Rimoin 1978).

Despite the known HLA-linked predisposition for IDDM, there are some problems with respect to the mechanism of conferring disease susceptibility which remain unresolved. A single genetic aetiology in the development of IDDM is not supported by monozygotic (identical) twin studies which have demonstrated a low concordance rate for IDDM. Furthermore, a wholly genetic role is further questioned by population studies which have indicated that HLA-DR4 occurs in 56-60% of the normal Caucasian population, whilst only 0.25-0.35% of the population progress to develop IDDM. These results suggest that although genetic aspects do play an important part in the aetiological development of IDDM; genetic susceptibility is not sufficient by itself for the progression to clinical IDDM. Presumably other mechanisms such as environmental factors or viral infections convert genetic susceptibility into clinical disease.

1.6 IMMUNE RESPONSES

It is now proposed to give a brief background information on the immune system in general, and to introduce some of the techniques employed in this study.

The Immune System

Lymphocytes are the major components of the immune system and play critical roles in various immunological responses. Recently, rapid progress has been made through the use of specific monoclonal antibodies for receptors / antigens on the surface of lymphocytes, and it has become apparent that lymphocytes are a heterogeneous group of cells. The major subpopulations of lymphocytes are the T- and B-lymphocytes. The T-lymphocytes are processed and differentiate under the influence of the thymus, and these cells constitute 80% of circulating lymphocytes in the peripheral blood. T-cells are further subdivided into two major categories i.e. immunoregulatory cells (suppressor and helper cells) and effector T cells. The immunoregulatory cells determine the tempo and magnitude of an immune response, whilst the effector cells are responsible for cell mediated immunity (CMI), including defense against bacterial and viral infections, allograft rejection and tumour immunity. B-lymphocytes are thymus independent and differentiate and mature in the foetal liver and bone marrow.

During their development, both T and B-lymphocytes express antigen specific receptors. Following stimulation by a specific antigen, the cells which recognise the antigen are activated by clonal selection with the help of non-specific accessory cells. Activated cells then differentiate as clones into (1) effector cells i.e. cytotoxic T-cells and antibody-secreting cells (mature B cells) and (2) memory cells constituting the

memory cell pool. Effector cells are short lived, but memory cells recirculate through the tissues and lymphoid organs via the blood and thoracic lymphatic duct, providing long-lived protective surveillance of the body from invading micro-organisms.

Mitogen induced lymphocyte responses

Lymphocytes proliferate when stimulated by a variety of agents including several plant lectins. Lectins are plant proteins that bind and cross-link specific cell surface glycoprotein determinants. The constitutive elements of bacterial cell membranes (e.g. lipopolysaccharide) also bind and activate cells in a similar fashion. These mitogens activate lymphocytes on a polyclonal non-immune basis (i.e. non-specifically), but the subsequent intracellular changes and lymphocyte differentiation are similar to those observed following antigenic stimulation.

Several mitogens selectively stimulate different lymphocyte subpopulations. For example, conconavalin A (conA), a lectin from the Jack bean Canavalis, stimulates both immature and mature thymocytes, whereas phytohaemagglutinin (PHA), a lectin from the red kidney bean Phasiolus vulgaris, stimulates mainly mature thymocytes, the response of immature thymocytes being very weak (Kimbal 1983). Some mitogens, for example bacterial lipopolysaccharide (LPS) and bacterial lipoproteins, are specific for B-lymphocytes. The selectivity of mitogen-induced stimulation has allowed immunologists to further characterise lymphocyte subpopulations.

B-lymphocyte immune responses

B-lymphocytes, the effector cells of humoral immunity, serve principally as

precursors for antibody forming cells.

Following primary challenge, by a thymus dependent (TD) antigen such as xenogeneic red blood cells, a normal antibody response results. However, primary antibody responses to TD antigens are dramatically reduced in mice which are thymectomized or congenitally 'athymic' (nude mice). Thus, activation of B-lymphocytes against a TD antigen also requires T-cell co-operation (Miller and Mitchell, 1968). Mosier and Coppleson (1968) demonstrated, using in-vitro cultures, that macrophages were also required for generation of primary immune responses. Furthermore, studies have found that major histocompatibility complex (MHC) antigens are also involved in the T-B cell co-operation (Rosenthal and Shevach, 1973). Thus, it is believed that immunogenic substances from the antigen are taken up by antigen presenting cells (APC), often macrophages, which express MHC gene products on their surface (Hirschberg et al 1983). The antigen is processed and displayed on the surface of the APC in association with MHC gene products, it is then presented to helper T (T_H) cells specific for the antigen in conjunction with MHC gene products (Thorsby et al 1982). This reaction results in expansion of T_H cells, which produce mitogenic and differentiative factors, which are able to activate T effector cells or B lymphocytes which expand rapidly in number and produce differentiated effector cells..

On the other hand, a group of antigens which are known as thymus independent (TI) antigens are able to activate B-lymphocytes to differentiate into antibody secreting plasma cells in T-cell depleted or nu/nu mice. Such TI antigens are mostly bacterial

polysaccharides e.g. E coli lipopolysaccharide (LPS) and type III pneumococcal polysaccharide (Hobart and McConnel, 1975).

Delayed-type hypersensitivity

T-lymphocytes involved in delayed-type hypersensitivity (DH) reactions have been shown to play critical roles in graft rejection, graft versus host disease, autoimmunity and immunity to intracellular parasites and tumours (Crowle, 1975). Therefore, elucidation of the mechanisms involved in induction and regulation of DH reactions are important to the understanding of these disease processes. Robert Koch first described DH in tuberculosis infected guinea pigs (Landsteiner and Chase, 1942). An inflammatory reaction was observed when the infected guinea pigs (i.e. tuberculin sensitised) were challenged with a lipoprotein antigen derived from tubercilli.

Four types of delayed hypersensitivity are recognised: Jones-mote, contact, tuberculin-type and granulomatous (Roitt et al 1985). DH reactions differ from antibody-mediated (immediate) hypersensitivity, in that the development of the immune response is slower. It cannot be transferred from one animal to another by serum, but it can be transferred by sensitised T-lymphocytes bearing a variety of defined surface phenotypes (Huber et al 1976; Miller and Jenkins 1987). The T cells necessary for DH are commonly called T_{DH} cells.

Contact sensitivity is a short lived form of DH, the skin lesion is usually maximal at 48 hrs, both in sensitised humans and experimental animals. Histological observations of the lesions show that the raised epidermis is characteristically infiltrated by mononuclear cells (T cells and macrophages). A number of chemicals will produce

dermatitis when applied to the skin. These include certain dyes (Phanuphak et al 1974), dinitrofluorobenzene (DNFB) and trinitrochlorobenzene (TNCB). The small haptens which induce DH are not antigenic by themselves due to their low molecular weights. However, DNFB or TNCB traverse the skin and then become conjugated with epidermal cell proteins. These antigens are processed, and presented by APC to T_H cells as described previously. Expansion of T_H cells is then able to stimulate precursor T_{DH} cells (effector cells involved in DH) to divide and differentiate into mature T_{DH} cells (Vadas et al, 1976). Further details of lymphocyte activation following mitogen activation, antigen stimulation or the events involved in DH are beyond the scope of this thesis, excellent reviews can be found elsewhere (e.g. Volpe 1981).

1.7 ANIMAL MODELS OF DIABETES

Diabetes mellitus is not a single disease, but rather a highly heterogeneous disorder with hyperglycaemia as the common denominator (Fajans et al 1978; Nerup and Lernmark 1983). Despite the broad categorisation of diabetes mellitus into types I and II, each form comprises many different subtypes which are manifested differently. These disorders also afflict a wide range of animal species (Cameron et al 1972; Coleman 1982). Furthermore, the diabetic syndrome manifests itself differently between each animal species and, by analogy with human diabetes, there are variations within a single animal species. Consequently, no single animal model will precisely duplicate every feature found in human diabetes. However, the use of several such models may lead to a greater understanding of human and animal diabetic disorders.

There are various protocols for inducing diabetes in animals, the earliest form being surgical pancreatectomy (Von Mering and Minkowski 1889). Since this method incapacitates the exocrine funtion of the pancreas, and morphological observation of the pancreas cannot be made, it has clear limitations as an experimental model. More recently, these methods have been superceded by chemical methods, which have provided specific destruction of the pancreatic islets whilst preserving exocrine funtion. These diabetogenic chemicals include alloxan, streptozotocin and N-nitrosocompounds (Dulin and Soret 1979). Diabetes produced by these methods is permanent, and many other metabolic abnormalities associated with diabetes are also found. One protocol devised by Like and Rossini (1976), that of multiple low dose streptozotocin (MSZ) treatment in mice, is particularly relevant to human IDDM

because insulitis is found in the islets.

Work presented in this study is concerned with diabetes induced by MSZ treatment. However, it is important to mention here that there are other animal models in which diabetes occurs spontaneously. These include the Bio-Breeding Wistar (BB/W) rat (Nakhooda et al 1977 and 1978) and the non-obese diabetic (NOD) mouse (Makino et al 1980).

1.8 PURPOSE OF STUDIES

The purpose of the studies presented in this thesis was to investigate the immune responses of MSZ induced diabetic mice (Like and Rossini, 1976), and to determine whether autoimmune reactions might be involved in the induction of the disease. Preliminary studies with MSZ treated mice have suggested that some immune responses may be defective following onset of diabetes (Buschard and Rygaard 1978a; Kim and Steinberg 1984), and furthermore, others have suggested that autoimmune reactions may be important in the induction of diabetes (Bure et al 1980; Kolb-Bachofen 1983; Kolb 1987). In contrast however, there have been reports which suggest that the defective immune responses observed following MSZ treatment in mice might not be associated with diabetes (Leiter 1982; Leiter et al 1983). Others suggest that autoimmune reactions are not required for the induction of diabetes (Bonnevie-Nielsen 1981). A more thorough characterisation of MSZ induced diabetes may therefore provide a clearer understanding of the induction of diabetes. Furthermore, if the lesions following MSZ treatment are similar to those observed in IDDM, this may provide an experimental model to study abnormalities of immune responses in human diabetes.

The investigation protocol presented here can be divided into three main sections. In the first section attempts were made to establish persistent diabetes in a given strain of mice using MSZ treatment. Induction of diabetes was assessed on the basis of several metabolic abnormalities including the presence or absence of hyperglycaemia, hypoinsulinaemia, glycosuria, ketonuria and weight loss. Some studies were also made on mice in which diabetes was induced following a single large dose of SZ

(SSZ), (see Dulin and Soret 1979). Secondly, a range of cellular and humoral immune responses were assessed in-vivo and in-vitro following experimental induction of diabetes. Attempts were made to study the effects of insulin treatment on some of the immune disturbances observed. The last section was concerned with the possible involvement of autoimmune factors in the aetiology of MSZ induced diabetes. The involvement of autoimmune reactions was assessed by applying some of the criteria previously described for other autoimmune disorders.

CHAPTER 2 INDUCTION AND ASSESSMENT OF DIABETES

2.1 INTRODUCTION

There is a growing body of both experimental and epidemiological evidence to support the involvement of environmental factors in the aetiopathogenesis of IDDM (Craighead 1978; Cahill and McDevitt 1981). In some instances the environmental factor may be chemical, and of particular interest are a class of chemicals called N-nitroso compounds. These compounds are widely used agricultural pesticides, and are ubiquitous environmental pollutants. N-nitroso compounds have been commonly found in the atmosphere and human food and have subsequently been implicated as a causative factor of human IDDM (Karam et al 1980; Calesnick 1980; Helgason and Jonasson 1981). Helgason and Jonasson (1981) reported that N-nitroso compounds present in Icelandic smoked cured mutton (a traditional delicacy eaten during the Christmas festivities in Iceland) may be responsible for causing diabetes in male children. The authors suggested that this may be due to the Icelandic cured mutton eaten by parents at or around the time of conception. The same group went on to reproduce similar results in the progeny of mice fed on Icelandic cured mutton (Helgason et al 1982). The mechanisms involved however are not known but the studies on mice suggested that paternal as well as maternal influences on germ cells were important in the induction of diabetes in the offspring. The best known of these compounds is the naturally occurring antibiotic Streptozotocin (SZ) biochemically characterised as 2-deoxy-2-(3 methyl-3-nitrosourea)-D-glucopyranose (White 1963). SZ consists of a nitrosoamide methylnitrosourea bound to the C-2 position of glucose. In an attempt to clarify the mode of action of postulated environmental factors

involved in the pathogenesis of IDDM, a considerable body of evidence has accumulated concerning the mechanisms by which SZ causes β-cell destruction.

SZ was isolated from the soil bacterium Streptomyces achromogenesis and was found to have antimicrobial properties against a wide spectrum of organisms (Lewis and Barbiers 1959; Varva et al 1959). Antitumour screening tests demonstrated a positive action, which created an interest in the compound as an antitumour agent (Weiss, 1982). During the preclinical in-vivo evaluation as an antitumour drug it became apparent that SZ produced hyperglycaemia within a few hours of treatment in rats and dogs (Rakieten et al 1963; Evans et al 1965). This unexpected finding was investigated further and SZ was found to produce permanent hyperglycaemia together with diabetes mellitus-like symptoms, including glycosuria, polyuria, ketoacidosis and weight loss. Furthermore, diabetes could be induced in a variety of animals including rat, dog, hamster, monkey, guinea pig and mouse. The diabetogenic dose of SZ varies between species, for example, mice are less susceptible to diabetes than rats (Cooperstein and Watkins 1981). The route of administration of SZ was also found to affect its efficacy, since antitumour trials indicated that five to six times more drug was required orally to produce the same effect as that achieved by an intravenous (IV) dose. However, subcutaneous and intraperitoneal injections were also effective (White 1963).

Following a single large IV dose of SZ in fed mice, a triphasic blood glucose response is produced. After an initial 45 minute delay hyperglycaemia is observed, peaking around 2 hours after injection. This phase is followed by marked hypoglycaemia which lasts for approximately 15 hours. A permanent hyperglycaemic phase is then attained by 48 hours (Junod et al 1967).

The mechanism of the early hyperglycaemic phase is unclear, although it has been suggested that it may be due to the inhibition of insulin release with the subsequent result of raised plasma glucose levels, since, in-vitro studies on isolated islets treated with SZ indicated that they released less insulin than normal islets in response to glucose (Bolaffi et al 1986). On the other hand, it was shown that SZ injection in-vivo did not significantly lower the concentration of insulin in the plasma (Junud et al 1969). The involvement of the liver was suggested, since a depletion of liver glycogen occurred 30 minutes before the peak of hyperglycaemia (Rerup and Tarding 1969; Rerup 1970). Furthermore, SZ-treatment increased levels of free fatty acids (FFA) in the plasma, and treatment with antilipolytic agents reduced the amounts of FFA (Cooperstein and Watkins 1981). This suggested that glycogen was being broken down to FFA which subsequently may lead to hyperglycaemia. Although the mechanisms by which SZ mobilises glycogen remain unclear, the role of liver glycogen is supported by the increased levels of FFA. Treatment with antilipolytic agents which prevented the increase in FFA levels, also prevented similar levels of hyperglycaemia as that achieved by SZ treatment alone. It is a possibility, therefore, that a combination of direct toxicity at the β-cell level and an indirect mechanism involving the mobilisation of liver glycogen are responsible for the hyperglycaemia

produced following SZ injection.

The hypoglycaemic phase following SZ injection can be explained by insulin released into the circulation from damaged and dying β-cells. Measurements of plasma insulin levels shortly before and during the hypoglycaemic phase show them to be increased (Dulin and Soret 1979). Furthermore, injection of anti-insulin serum attenuates the hypoglycaemia, presumably by binding the released insulin before it can affect glucose levels. (Junod et al 1967; Rerup and Tarding 1969). As a result of the loss of β-cells, a permanent hyperglycaemic phase is attained after the hypoglycaemic effect of insulin from damaged β-cells diminishes.

Morphological changes following SZ injection in mice show that the β-cells exhibit gross changes which ultimately lead to death. The earliest morphological changes occur after 1 hour, these include hypertrophy of golgi apparatus and occasional pycnosis of nuclei of the β-cells (Dulin and Soret, 1979). Within 1.5 hours, the numbers of intramembranous particles of the plasma membrane decrease (Orci et al 1976). This is followed by clumping of nuclear chromatin and decreased nucleolar size at around 2 hours (Lazarus and Shipiro 1982). Extensive pycnotic nuclei and disruption in the membranes of secretary granules is observed after 3-4 hours (Wilander 1975).

When mice are given a single bolus dose of SZ (200 mg/kg body weight),hereafter refered to as SSZ. SSZ treatment produces hyperglycaemia which occurs within a few hours, this is most likely due to the destruction of β-cells. Morphological examination of the islets of Langerhan shows that within a few hours gross disruption of the *B*-

cell is present (Orci et al 1976). By contrast, however, if mice are injected daily with multiple subdiabetogeneic doses of SZ (40 mg/kg body weight) for 5 consecutive days (hereafter referred to as MSZ), hyperglycaemia appears slowly. Frank hyperglycaemia occurs around 10 days after the initial injection (Like and Rossini 1976).

Several studies have found that following MSZ-treatment in mice there is a permanent morphological and functional impairment of insulin release (e.g. Like and Rossini) However, some compounds have been shown to prevent the diabetic syndrome caused by SZ treatment . High levels of glucose and nonmetabolisable analogues of glucose 3-0-methyl glucose and 2-deoxyglucose have been shown to prevent diabetes in MSZ treated mice (Rossini et al 1977c and 1978b). Another study found that following injections of equivalent concentrations of SZ or methylnitrosourea (MNU), SZ was detected at much higher levels in the islets than MNU (Anderson et al 1974). These data have suggested that the glucose moiety renders diabetogenic N-nitroso compounds with a specificity for the β cell. Further studies have shown that administration of inhibitors of poly(ADP-ribose) synthetase, and scavengers of free oxygen radicals also prevented the induction of diabetes following SZ treatment in mice (For review see Le Doux et al 1986). These studies have contributed to proposals regarding the mechanism of action of SZ, which are described below.

It has been proposed that SZ may cause its toxicity by fragmenting DNA. The activity of poly (ADP-ribose) synthetase (a DNA repairing enzyme) is greatly activated during DNA repair (Wilson et al 1984; Sandler and Swenn 1985; LeDoux et al 1986), and it

has been shown that the activity of this enzyme is also increased following incubation of islets with SZ in-vitro (Yamamoto et al 1981). Levels of nicotinamide adenine dinucleotide (NAD), a substrate of the above enzyme, have been reported to be lowered in SZ-treated islets, and adminstration of nicotinamide was shown to prevent diabetes (Dulin and Wyse, 1967). It might be that the level of (ADP-ribose) synthetase is activated to such an extent (in an attempt to repair the DNA) that NAD levels become critically depleted, and thereafter the β-cell is unable to function normally and dies.

A single SZ injection in male mice produced severe diabetes whilst female mice remained euglycaemic (Maclaren et al 1980). MSZ treatment in male mice produced diabetes, but female mice of the same strain were resistant (Rossini et al 1978; Kroman et al 1981 and 1982; Leiter 1982). The incidence of IDDM has been shown to be influenced by sex. In man incidence studies have shown that more males than females develop IDDM in the age group 10-20 years (De Beaufort et al 1988).

To determine if the differences in susceptibility to hyperglycaemia induced by MSZ-treatment was due to sex hormones, Kim and Moody (1984) treated mice with female or male hormones prior to MSZ-treatment. Oestrogen treatment in males prevented diabetes whilst androgen treatment in females, which would not normally become diabetic following MSZ-treatment, produced diabetes. These results demonstrate that sex hormones influence the induction of hyperglycaemia in MSZ-treated mice. The exact mechanisms involved are unclear. However, it is known that oestrogen inhibits immune responsiveness (Kim and Moody, 1984) and it could be that oestrogen

prevents SZ-induced hyperglycaemia in female mice or oestrogen treated male mice by immunosuppression. But it must be borne in mind that even in-vitro, in the absence of hormonal influences isolated islet cells from male mice are more susceptible to SZ treatment than female islet cells (Kroman et al 1981 and 1982). It is a possibility, therefore, that both human and experimentally induced diabetes in animals, are influenced by common mechanisms that are under the control of sex hormones, and MSZ-induced diabetes in mice may be a useful model to further investigate the role of sex hormones in the aetiology of IDDM.

By analogy with the involvement of MHC in the development of IDDM in man (Curdworth and Wolf 1982) a strain dependent sensitivity to both single SZ and MSZ induced diabetes has also been reported in laboratory animals (Rossini et al 1977a and 1977b; Kroman et al 1979). It was observed that LAF mice, although sensitive to SSZ-treatment, were not susceptible to MSZ-induced diabetes. (Kim and Steinberg, 1989). Further complications have also been reported, Rossini's group (Rossini et al 1977) reported that BALB/c mice were resistant to MSZ induced diabetes, however, hyperglycaemia was reported in BALB/c BOM mice (originally derived from BALB/c mice, Paik et al 1982). The apparent discrepancy between these results may be due to genetic variation or environmental factors.

In light of the strain dependency in the induction of MSZ-induced diabetes, the purpose of the work presented in this chapter was to investigate whether permanent and reproducible diabetes-like syndrome could be induced by MSZ treatment in several strains of mice. Diabetes was assessed by several physiological measurements, thus the presence of hyperglycaemia, hypoinsulinaemia, glycosuria,

ketonuria and changes in body weight were investigated following MSZ treatment. Some of the investigations were also carried out in SSZ treated mice.

2.2 MATERIALS AND METHODS

2.2.1 Reagents

Analytical grade reagents and double distilled water were used throughout. The chemicals and their sources were as follows:

N-2-hydroxyethyl-piperazine-N-2 ethansulphonic acid (HEPES), D-glucose, powdered RPMI 1640, dimethylsulphoxide (DMSO), streptozotocin (SZ) and powdered Hanks balanced salt solution (HBSS), were purchased from Sigma Chemicals Co. Ltd., U.K.

Thiomersalate was purchased from BDH Chemicals Ltd., U.K.

Porcine monocomponent insulin was purchased from Novo Research Institute, Bagsvaerd, Denmark.

Na[^{125}I] (IM530, 100mCi/ml) was obtained from Amersham International U.K. Bovine serum albumin (BSA) fraction V was obtained from Miles Laboratories Ltd. U.K.

Human insulin standard RD13 and binding reagent RD12 were purchased from Wellcome Diagnostic Reagents Ltd. U.K.

Foetal calf serum (FCS), penicillin, streptomycin and trypsin/EDTA were purchased from Flow Laboratories Ltd. U.K.

All tissue culture plastics were purchased from either Costar Cambridge Mass. USA, Gibco U.K. or Sterilin U.K.

2.2.2 Animals

Male albino Tylers original (TO) outbred strain mice, and BALB/c conventional mice were purchased from Bantin and Kingman, U.K. Homozygous lean mice (HO) were obtained from the littermates of Aston obese mice (ob/ob), maintained at Aston University, Birmingham U.K. The obese mice are derived from C57BL/6J ob/ob mice and their origin has been described in detail elsewhere (Bailey et al 1982). MF1 (outbred strain) were also from the colony maintained at Aston University.

The animals were maintained in air conditioned rooms at 22 ± 2°C with a lighting schedule of 9.5 hours light (0900-1730) and 14.5 hours dark. Mice were allowed free access to tap water and standard pellet diet (mouse breeding diet, Heygate and Sons Ltd. U.K.). Food was removed for 3 hours prior to injection of SZ at 1200 hours. Animals were housed in cages, 5 animals per cage, and the cages were cleaned three times a week.

2.2.3 Induction of diabetes

10 week old male mice (25 ± 2g) were used throughout. One group received an intraperitoneal (IP) injection of SZ (200 mg/kg body weight). This group will be referred to as single streptozotocin (SSZ) treated mice. Another group received an IP injection of SZ (40 mg/kg body weight) per day for 5 consecutive days. This group will be referred to as multiple streptozotocin (MSZ) treated mice. Although an intravenous injection of SZ is known to be more effective because of the short half life of SZ (Weiss, 1982), IP injections were chosen here to conform with the established techniques of Like and Rossini (1976) and furthermore, IP injections are a more

convenient way of administering SZ treatment. SZ was dissolved in ice cold citrate buffer (prepared as described in appendix A1), pH 4.5, immediately before injection. Control mice received similar (either a single or five) injections of citrate buffer only. The time period referred to in the text is the number of days from the first injection of SZ (days).

2.2.4 Physiological methods

Blood samples (50µl) were collected from the cut tip of the tail from conscious non-fasting mice. Plasma glucose was determined by an automated glucose oxidase procedure (Stevens 1971) using a Beckman Glucose Analyser (Beckman Ltd. U.K.). The remaining plasma was stored at -20°C for insulin radioimmunoassay (Hales and Randle 1963). Glycosuria and ketonuria were tested for by using Labstix® (Miles Laboratories Ltd. England). When handled mice frequently urinate spontaneously and urine samples were applied directly to Labstix. Levels of glucosuria and ketonuria were scored as positive if they were in excess of 2.2 mMol/l and 0.5 mMol/l.

2.2.5 Insulin Radioimmunoassay

Radioimmunoassay is dependent upon the competition between labelled and unlabelled antigen for binding sites on specific antibodies (Rodbard 1973). Increasing amounts of unlabelled antigen in a sample produce a proportional decrease in the binding of labelled antigen to the antibody (Yalow and Berson 1960). The level of radioactivity associated with the antibody antigen complex is related to the concentration of unlabelled antigen in the original sample. In the present study, insulin was measured using the double antibody technique of Hales and Randle (1963), with

slight modification in that bound (guinea pig anti-porcine insulin precipitated with rabbit anti-guinea pig globulin) and free ^{125}I labelled insulin were separated by centrifugation.

^{125}I labelled insulin was made using ^{125}I using the chloromine-T method of Hunter and Greenwood (1962). This method was routinely employed in our laboratory for economic and sufficient generation of high quality ^{125}I for insulin radioimmunoassays.

2.2.6 Preparation of reagents for insulin radioimmunoassay

1. Diluent buffer. The following reagents were added to 1 l of double distilled water 6.2g sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$), 0.25g thiomersalate (as a preservative) and 5.1g of BSA. The pH was adjusted to 7.4

2. Insulin binding reagent (RD 12). The lyophilised reagent consisted of guinea pig anti porcine insulin serum precipitated with rabbit anti guinea pig globulin. The reagent was reconstituted in 8ml of double distilled water on the day of the assay.

3. ^{125}I-iodinated insulin. ^{125}I-insulin was prepared to an average specific activity of 250 μCi/μg using the chloramine T method. The specific activity (of a 50-200 μl aliquot) was reduced to 50 μCi/μg by the addition of a calculated amount of 'cold' unlabelled porcine insulin. The count rate was reduced with diluent buffer such that a 50 μl aliquot used in the assay contained 9,500-11,000 cpm.

4. Insulin standards. The contents of 1 bottle of Wellcome human insulin standard RD13 (25-35 μU) was diluted to 1 μU/ml with diluent buffer. 0.2 ml aliquots of this solution were dispensed into LP3 tubes and stored at -15°C. When required on the day of the assay one of these tubes was diluted with 0.8 ml of diluent buffer to

provide a top standard insulin concentration of 200 μU/ml. Five further serial dilutions were made from this stock standard tube to provide a range of standard insulin concentrations from 6.25-200 μU/ml.

In the present study human insulin standard was used to determine the amounts of immunoreactive insulin present in the plasma of mice and that secreted from hamster derived HIT-T15 cells (see Chapter 6). The reasons for this were three fold. Firstly, human insulin standard was more readily available than mouse or hamster insulin standard. Secondly, the activity of the human insulin standard was found to be similar to rat insulin (rat insulin is identical to mouse insulin). See Appendix 3. Thirdly, the present study was not concerned with the measurement of absolute levels of insulin but rather was aimed at estimating the relative amounts of insulin in normal and diabetic mouse serum or to determine the changes in the level of insulin released from HIT-T15 cells. Consequently, all measurements of insulin are expressed in terms of human insulin equivalents.

2.2.7 Procedure for the radioimmunoassay of insulin

Assay reagents were added to LP3 tubes in the order indicated in the reaction protocol summarised in Table 2.1 below.

Table 2.1 Protocol for the addition of reagents in the double antibody radioimmunoassay of insulin

Tube description	Tube number	Initial reactants	Binding reagent	Iodinated insulin
Total counts	1-3	-	-	+
Blank	4-6	Buffer	Buffer	+
	7-9	6.25	+	+
Insulin	10-12	12.5	+	+
standards	13-15	25.0	+	+
μlU/ml	16-18	50.0	+	+
	19-21	100.0	+	+
	22-24	200.0	+	+
Unknown samples	24-n	+	+	+

All reactants were added in 50 μl aliquots

Insulin standards, total counts and blanks (containing buffer instead of standards and binding reagent) were assayed in triplicate. 50 μl aliquots of insulin standards, human and unknown samples, were transferred to LP3 tubes. A 50 μl aliquot of insulin binding reagent was added to all tubes (except totals and blanks). The contents of each tube were vortex mixed without frothing, and incubated at 4°C for 4 hours. A 50 μl aliquot of ^{125}I-insulin (9,500-11,000 cpm, specific activity 50 μCi/μg) was then added to all tubes. After gentle vortex mixing, all tubes were incubated for a further 16-18 hours at 4°C. After this second incubation 0.5 ml of diluent buffer was added to all tubes except the totals and the contents vortex mixed. Free and antibody bound ^{125}I-insulin was separated by centrifugation (1,500g Mistral MSE) for 30 minutes at room temperature. The supernatant containing free labelled insulin was carefully

decanted by inverting the tubes and the remaining drops of buffer aspirated from the rim of the inverted tube with the aid of a pasteur pipette connected to a vacuum pump. The tubes were then left at an angle of 30° for approximately 4 hours to dry at room temperature. The ^{125}I-activity associated with the precipitate was counted for 1 minute on a Compu-gamma gamma counter (LKB Instruments Ltd., Sweden).

2.2.8 Computation of insulin levels

The construction of standard curves and the computation of results was performed using an RIA package associated with the gamma counter. Log_{10} of the standard insulin concentration was plotted against the counts per minute in the bound fraction, and the sample unknown insulin concentration was determined automatically by direct extrapolation from the graph. Computation of the correlation coefficient and the minimal sensitivity was achieved using a BBC computer software package (see Lambert 1987).

2.2.9 Statistical analysis

Statistical analysis throughout this thesis was performed using the two tailed students t-test. Differences were considered to be significant when $p < 0.05$. Mean values were expressed as mean ± SEM.

2.3 RESULTS

2.3.1 Plasma Glucose

Plasma glucose levels in male TO mice were followed over a period of 70 days after MSZ treatment. The data is presented in Figure 2.1. Plasma glucose levels in MSZ treated mice increased progressively over a period of 40 days, from a preinjected level of 10 ± 1.5 mMol/l to an observed maximum level of 54 ± 7 mMol/l. The raised blood glucose levels in MSZ treated mice were significantly ($p < 0.05$) higher than those values observed in vehicle injected (control) mice. Control mice did not show any significant alteration in plasma glucose levels throughout the course of the study. A similar glucose response curve was produced by HO mice. BALB/c mice exhibited a transient hyperglycaemic phase around 18 days, however, the other strains tested displayed a permanent diabetic state (Table 2.2). SSZ treated TO mice also exhibited hyperglycaemia (Table 2.2) within 16 days of the injection.

2.3.2 Plasma Insulin

Table 2.2 shows the plasma insulin levels following MSZ treatment. 16 days after MSZ treatment, MSZ treated mice had significantly reduced insulin levels when compared to the control mice. However, insulin levels following SSZ injection gave the lowest values. All insulin levels are expressed as human insulin equivalents. For the determination of absolute levels of insulin in mice it would be necessary to use mouse insulin standard. However in this study human insulin was used because the aim of the study was to establish the difference in the insulin levels between diabetic and normal mice.

2.3.3 Glycosuria and Ketonuria

MSZ treated mice were examined for the presence of glycosuria and were found to be markedly positive, whilst the control mice gave negative results. However, there was no ketonuria in the MSZ treated mice when examined on day 16, even though the mice were hyperglycaemic and positive for glycosuria at this stage (Table 2.2).

2.3.4 Body Weights

Following SSZ injection in TO mice, the diabetic mice lost 28 ± 6% of initial body weight, over a period of 13 days, the control mice gained 13 ± 3% in the same period. MSZ treated mice did not show a net weight loss when measured over 17 days, however the weight gain was lower when compared to the control mice. Figure 2.2 shows that 6 days following MSZ treatment, MSZ treated mice had a significantly lower weight when compared to their initial weight. Subsequently, the weight loss in MSZ treated mice recovered and at the end of the study there was a net weight gain, but the gain in weight was still lower than the weight gain in control mice.

2.3.5 Fatality

4 out of 20 SSZ treated mice died within 13 days of the SSZ injection. There were no deaths in the control and MSZ treated mice (Table 2.2).

Table 2.2 Physiological Measurements After SSZ/MSZ Treatment

Treatment (strain of mice)	Plasma Glucose m mol/l	Plasma Insulin μU/ml	Body Wt % change of initial value	Glycosuria	Ketonuria	Number of Fatalities after 13 days of treatment
Control@ (TO)	10 ± 2	146 ± 31	16 ± 2	-	-	0 / 50
SSZ (TO)	43 ± 8*	7 ± 3*	-28.6*			4 / 20
MSZ (TO)	28 ± 4*	14 ± 3*	6 ± 3*	+	-	0 / 50
MSZ (MF1)	25 ± 3*					
MSZ (HO)	24 ± 3*					

Body weight changes were recorded on day 13 following start of streptozotocin treatment, all other measurements were performed 16 days after the treatment. @ There is no significant difference between the control values measured in to, MF1 and HO mice. + indicates presence,- indicates absence, * indicates $\rho < 0.05$, $n = 5$.

Figure 2.1

Plasma glucose of TO, HO and BALB/c mice following MSZ treatment. Plasma glucose values in control mice of each strain did not change significantly throughout the study from the initial level
* indicates $p<0.05$ when compared to control values n=5

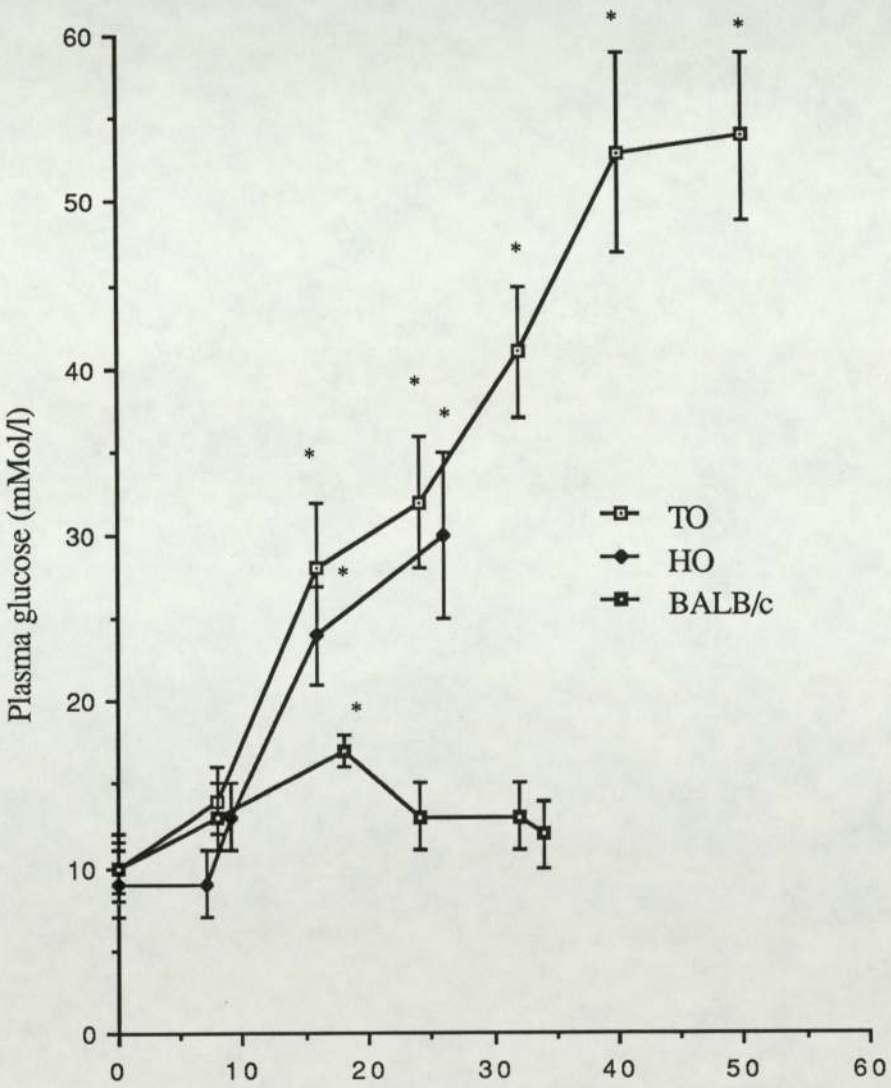
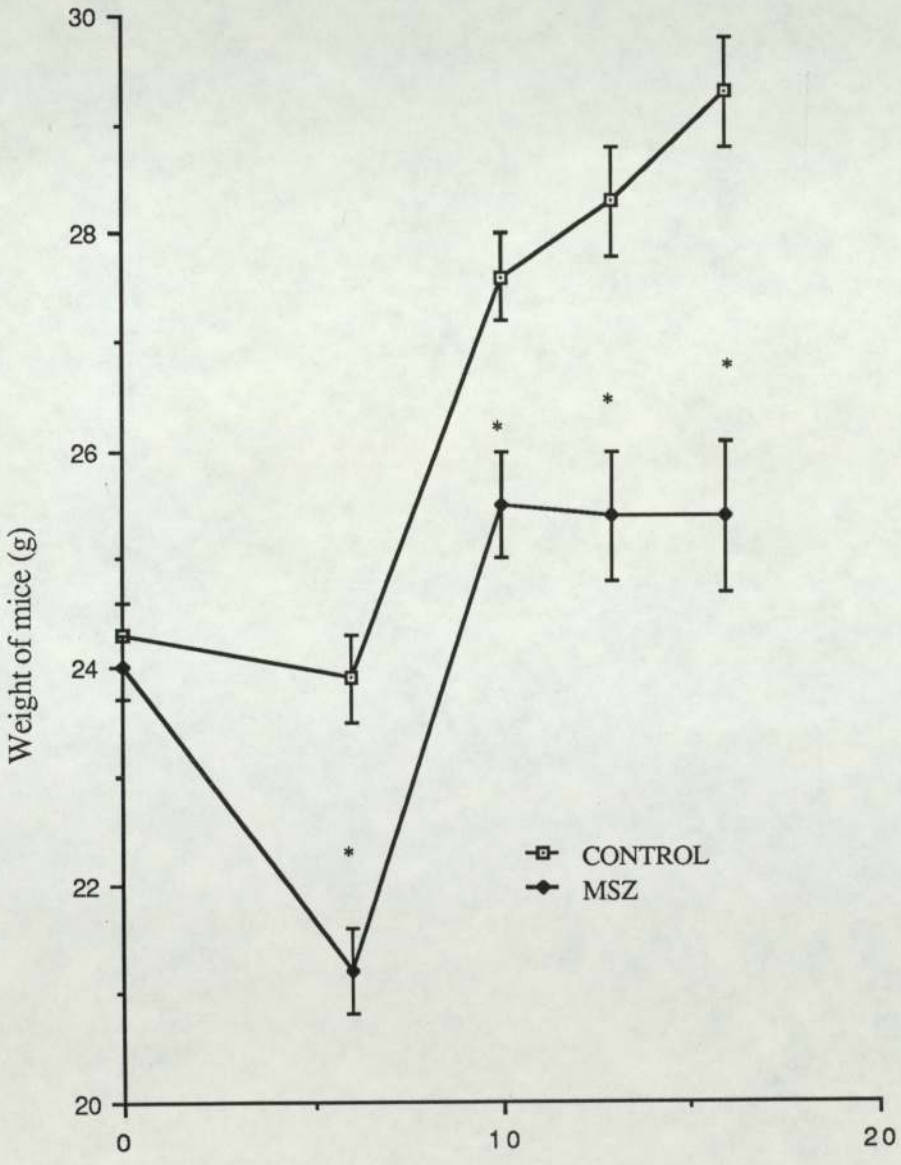


Figure 2.2

Changes in weight following MSZ treatment

* indicates $p < 0.05$, $n = 10$

in control and MSZ treated TO mice



2.4 DISCUSSION

The results presented here have shown that SSZ treatment produced hyperglycaemia in male TO mice. This observation is in accordance with observations reported from other strains of mice (Rerup 1970). However, there have been reports indicating that severe hyperglycaemia is not produced following SSZ treatment in some strains of mice (Rossini et al 1977a; Kim and Steinberg 1984). This suggests that MHC may be involved in the susceptibility of different strains of mice to SSZ induced diabetes. Work presented here on MSZ treated mice confirms this point. MSZ treatment produced permanent hyperglycaemia in TO, MF1 and HO mice. Permanent hyperglycaemia induced by MSZ treatment has been reported in other strains of mice by many authors (Like and Rossini 1976; Rossini et al 1977a and 1977b; Paik et al 1980; Kiesel et al 1983; Nakano et al 1984). By contrast, only a transient hyperglycaemic phase could be induced in male BALB/c mice. There have been reports that BALB/c mice were not susceptible to MSZ induced hyperglycaemia. (Kim and Steinberg 1984).

The involvement of genetic background in the susceptibility to MSZ induced diabetes is well established (Rossini et al 1977a; Kim and Steinberg 1984; Weber et al 1984). Kim and Steinberg (1984) reported that out of 6 inbred strains examined, only 3 produced the same level of hyperglycaemia as that observed in CD-1 mice. Studies on the effect of SZ treatment (consisting of 2 injections of SZ, 120 mg/kg body weight, IV, given 1 week apart) on recombinant strains of B10.BR mice, have shown that alleles on the H-2^k and Ia loci are associated with the susceptibility to SZ induced diabetes (Weber et al 1984). Thus, MHC genes also have an important role in

influencing the susceptibility of different strains to SZ induced hyperglycaemia.

Recently, it has been reported that SZ treatment on different inbred strains with the same MHC alleles did not produce the same level of hyperglycaemia, indeed some strains were not susceptible (Weber et al 1984). MSZ treated on BALB/c mice from the Jackson Laboratories were resistant to the induction of hyperglycaemia, whilst the mice purchased from two other sources were susceptible (Kim and Steinberg 1984). These results suggest that genetic background other than MHC genes, and/or environmental factors, also influence the susceptibility to MSZ induced hyperglycaemia. The nature of these factors is unknown. However it is believed that SZ may kill the β-cell by activating poly (ADP-ribose) synthetase, whereby NAD levels are depleted critically and the β-cell dies (LeDoux et al 1986). One possible explanation for strain susceptibility, therefore, is that susceptible strains might possess higher enzymatic activity of poly (ADP-ribose) synthetase, or alternatively, the sensitive strains may be able to degrade SZ to a more toxic form.

In the present study, the MSZ induced hyperglycaemia in TO mice was associated with hypoinsulinaemia and glycosuria. However, MSZ treatment did not induce ketonuria in the diabetic mice. Nakano et al (1984) also reported that ketonuria could not be induced by MSZ treatment. Ketonuria can occur in uncontrolled IDDM and it has been reported in BB rat and NOD mouse (Nakhooda et al 1978; Makino et al 1980). The reason for the absence of ketonuria in MSZ treated mice may be due to the relatively mild diabetes following MSZ treatment, indeed Nakano et al (1984) reported that mice injected with higher doses of SZ (60 mg/kg daily over 5 days) did have

ketonuria. This observation gains support from the fact that, if diabetes is not controlled by insulin therapy in IDDM patients, BB rat or NOD mouse, the individuals die. However, MSZ treated TO mice were alive and healthy without insulin therapy 10 weeks after induction of diabetes.

SSZ treatment was fatal in 4 out of 20 mice and the diabetic mice exhibited severe weight loss by the end of 13 days. The effects of SZ and MSZ treatment on body weights are difficult to interpret in the absence of data on food and water consumption. Clearer information would be provided by paired feeding studies where food/water consumption and faeces/urine production are measured using metabolic cages. Despite this restriction, however statistical analysis revealed a significant difference between the effects of SSZ and MSZ treatment on body weight. A single dose of SZ may be cytotoxic not only towards pancreatic islets but also towards other organs. The death of SSZ treated mice is probably not due only to severe hyperglycaemia because MSZ treated mice with similar levels of hyperglycaemia were without apparent ill effects.

In conclusion, persistent hyperglycaemia associated with hypoinsulinaemia and glycosuria was observed in MSZ treated TO, HO and MF1 mice, but not in BALB/c mice. Together with previous reports by other authors, the work presented here has successfully demonstrated that MSZ treatment can induce a diabetic state in certain strains of mice. Subsequent studies in this thesis will use this MSZ induced model of diabetes to study possible alterations in the immune system of diabetic mice. Thus Chapter 3 examines cell mediated and humoral immunity in-vivo, whereas Chapter 4

concentrates on mitogen induced responses in-vitro. In Chapter 5 the function of natural killer cells and antibody dependent cell mediated cytotoxicity are examined and finally, in Chapter 6, studies were carried out to assess whether or not MSZ treated diabetic mice showed signs of autoimmune reactivity.

CHAPTER 3 IN-VIVO IMMUNOLOGICAL ABNORMALITIES IN MSZ TREATED MICE

3.1 INTRODUCTION

In the past most of the immunological studies concerned with diabetes have been involved with the immunology of insulin and the problems of insulin resistance in insulin treated diabetics. Until recently, few researchers had devoted much interest to immunology and autoimmunity of diabetes mellitus. The immunology of diabetes can be arbitrarily divided into two major areas of study. One is concerned with the involvement of the immune system in the aetiology and pathogenesis of the disease. The other is concerned with the effects of the diabetic state on immune competence. The aim of the present chapter is to address the latter.

It has been established that patients with diabetes are more susceptible to infections such as tuberculosis, mycosis and staphylococci than normal subjects (Joslin et al 1959; Adamkiewicz 1963; Johnson 1970). It has also been reported that these infections tend to be more protracted and severe in diabetic patients (Editorial 1959).

Immune deficiencies are often invoked to explain the increased susceptibility to infections (Eliashiv et al 1978). It has been suggested that depressed cell mediated immunity may be involved, however, depressed lymphocyte numbers also contribute to diminished lymphocyte function. Impairment of lymphocyte numbers in poorly controlled diabetes has been reported by several authors (Brody and Merlie 1970; Cattaneo et al 1976; Hann et al 1976; Selam et al 1979). Examination of the thymus

glands from IDDM patients has shown that the number of Hassal's corpuscles (presumed sites of lymphocytic death and breakdown) were increased and that the size of the largest corpuscles were significantly decreased (Souadjian et al 1970). The authors also reported that epithelial cells from the thymic medulla were smaller in diabetic patients. The association of diabetes with changes in lymphoid organ weight and cellularity has prompted numerous investigations in various animal models of diabetes. In studies with alloxan and SSZ treated mice, involution and atrophy of the lymphoid organs has been reported (for review see Drell and Notkins 1987). However, the validity of these results, as well as their relevance to human diabetes, depends on whether they occur as a direct result of endocrine disturbance and/or hyperglycaemia, or are toxic side effects of the drugs used to provoke the diabetic state.

3.1.2 Humoral Immunity

Antibody formation following in-vivo immunisation with bovine serum albumin (a thymus dependent antigen) has been reported to be normal in alloxan diabetic mice (Dolkart et al 1971). By contrast there have been several reports to suggest that humoral immunity to another T dependent antigen, sheep red blood cells (SRBC), is depressed in alloxan treated mice (Ptak et al 1977; Pavelic et al 1978; Maduna et al 1978; Slijepcevic 1979; Barg et al 1980; Gaulton et al 1985). Antibody responses to SRBC have also been reported to be depressed in MSZ treated mice (Kim and Steinberg 1984; Nakano et al 1984). In SSZ induced diabetes a depressed anti-SRBC plaque forming cell response has also been observed (Ishibishi et al 1980; Saiki et al 1980; Handwerger et al 1984; Gaulton et al 1985). Similarly in MSZ-treated mice

anti-SRBC plaque forming cell responses have recently been reported to be diminished (Kim and Steinberg 1984; Nakano et al 1984). In contrast however, Barg et al (1980) previously reported that anti-SRBC plaque forming cell responses were similar to those values detected in control mice.

Antibody formation against thymus independent (TI) antigens has also been examined in alloxan treated mice, where reports have indicated that antibody formation against microbial antigens was either impaired (Sood et al 1975; Ptak et al 1977; Barg et al 1980) or unaltered (Maduna et al 1978). Similarly, contrasting findings have been observed in SSZ treated mice, Kitahara et al (1981) and Busby et al (1983) found that B-cell function in antibody production against bacterial TI antigen was impaired, however Saiki et al (1980) found that it was intact.

The work on humoral immunity in SSZ or alloxan treated mice has indicated that the ability to form antibodies to some antigens is depressed, whilst to other antigens it is intact. Thymus dependent antigens require T and B lymphocytes and macrophages to co-operate in order to produce a significant immune response. Since some of the studies with antigens suggest that antibody responses are intact in the diabetic mouse and the fact that T-lymphocytes are not involved in the antibody response to TI antigens, it is possible that B-lymphocyte function is intact and that the immunological defect lies in the T cell or macrophage populations. To investigate whether cell mediated immunity is defective in diabetes, researchers have used various in-vivo studies including, delayed type hypersensitivity (DH) tests, granuloma formation, allograft rejection and tumour rejection.

3.1.3 Cell mediated immunity

Mahmoud et al (1976) found that granuloma formation against S.mansoni eggs was depressed in SSZ treated mice. The authors found that DH, as measured by footpad swelling, was also depressed against the same antigen. Depressed footpad swelling has also been reported in SSZ treated mice against SRBC (Ishibishi et al 1980) and the hapten azobenzene carsonate (Gaulton et al 1985). Contact sensitivity to DNFB or oxazolone was found to be diminished in SSZ treated mice (Saiki et al 1980; Nichols et al 1981) and other MSZ-treated mice (Barg et al 1980). Other investigators have reported that skin allograft rejection time was increased (Mahmoud et al 1976) and tumour rejection impaired (Nichols et al 1979). Similarly, in alloxan treated mice, CMI has also been found to be diminished (for review see Drell and Notkins 1987).

It can be seen that both humoral and cell mediated immunological functions are sometimes depressed in SSZ or alloxan treated mice. The exact mechanisms underlying these observations are unclear. One possible explanation for the depression may be due to the insulinopenic state of the diabetic mice. This proposal has been supported by studies which found that the depressed humoral or cell mediated immunity could be reversed by insulin (for review see Drell and Notkins 1987). However, the reversal of immunological responses has often been partial, and the fact that spontaneously occurring diabetes in obese, hyperinsulinaemic db/db and ob/ob mice show similar impairment of cell mediated immune responses (Handwerger et al 1984), does not lend support to the proposal. Further work is required to investigate the role of insulin on immune competence.

Whilst immunological functions are sometimes altered in chemically induced diabetic mice, it is apparent that there is considerable discrepancy in the literature. One problem in interpreting the results of previous studies on the effects of MSZ treatment on cellular and humoral immunity is that many of the experiments were carried out on different strains of mice. Thus strain differences in susceptibility to MSZ-treatment and environmental factors make it difficult to compare results. One aim of the present study therefore, is to examine humoral and cell mediated immunity in a single strain of mice. Work presented in this chapter investigates both humoral and cell mediated immune function in MSZ To treated mice. Furthermore, restoration of cell mediated immune function was also examined following insulin therapy in diabetic mice.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of thymus and spleen cells and measurement of cellularity

Lymphoid cells for in-vitro studies in this and the following chapters were prepared as follows. Aseptic techniques were used throughout. Briefly, mice were killed by cervical dislocation and surface sterilised using 70% ethanol. The spleen and thymus were removed and weighed. Cell suspension was prepared by teasing the organs with forceps in culture medium at 4°C. After removal of clumps and debris, the cell suspension was washed 3 times by centrifugation at 150g for 10 minutes in a refrigerated centrifuge. Cellularity of the lymphoid organs was assessed by counting spleen and thymus lymphocytes using a haemacytometer.

3.2.2 Determination of cell viability

The viability of cell suspensions was determined by trypan blue dye exclusion (Mishell and Shiigi 1980). Viability of spleen cells used in this study was always > 95%.

3.2.3 Immunisation of mice with sheep red blood cells (SRBC)

SRBC (Flow Laboratories, U.K.) were stored in Alsever's solution and washed in HBSS by centrifugation at 150g for 10 minutes at 4°C. MSZ treated and control mice were injected intraperitonealy (IP) with 0.5ml 10% v/v SRBC in HBSS 10 days after the 1st SZ injection.

Time periods given in the figures for the subsequent immunological tests involving

immunised mice refer to the number of days after the SRBC injection. The same batch of SRBC was used for the immunisation and the immunological tests.

3.2.4 Haemagglutination titre (HA)

Antibody titre against SRBC was measured in MSZ treated and control mice 7 days after SRBC injection. A standard haemagglutination technique was used as described elsewhere (Hudson and Hay 1980). Antibody titres were expressed as $-\log_2$.

3.2.5 Antigen reactive cells (ARC)

The immunocytoadherence assay provides a sensitive method for detecting antigen reactive cells (ARC). Mice were initially immunised with sheep red blood cells (SRBC). Lymphocytes from immunised mice with anti-erythrocyte surface receptors bind to SRBC to form 'rosettes', which consist of a central lymphocyte surrounded by bound erythrocytes. Such antigen binding cells are also referred to as rosette forming cells (RFC).

200µl of spleen cells at 2 x 10^6 cells/ml in HBSS and 200µl of 2% v/v SRBC in HBSS were incubated overnight at 4°C. After the incubation, the cells were gently resuspended by rotating the tube over ice. ARC were counted using a haemocytometer, a rosette was arbitrarily defined as a single lymphocyte binding 5 or more erythrocytes (Hudson and Hay 1980). The number of ARC was expressed as ARC/10^6 viable spleen cells.

3.2.6 Plaque forming cells (PFC)

The haemolytic plaque assay permits the visualisation of small amounts of lytic antibody released in the vicinity of a single lymphocyte. Lymphocytes and a dense suspension of indicator red blood cells are mixed and distributed as a monolayer. Secreted antibodies sensitise the indicator red blood cells around the lymphocyte. With the addition of complement, the red blood cells lyse and clear plaques appear (Jerne and Nordin 1963). In the present study, the method of Cunningham and Szenberg (1968) was used. This method has advantages over the agar plate method of Jerne. Firstly, sensitivity is increased by greater than three-fold over the Jerne type assay (Cunningham 1965). Secondly, it allows the cells in a monolayer to be examined at high magnification. Furthermore, the method is simple and economic to use.

The method described below, measures the number of cells secreting high efficiency IgM antibodies. This method measures only cells secreting IgM antibodies because, (i) IgM has a greater efficiency in fixing complement and (ii) titres of IgM and IgM producing cells both increase and peak at the same time, after a primary immunisation of SRBC (Hudson and Hay 1980).

An appropriate number of spleen cells producing approximately 100 PFC per chamber in a volume of 220 µl HBSS were added to 40 µl of SRBC absorbed guinea pig serum as a source of complement and 80 µl of 15% w/v SRBC in HBSS. The mixture was then warmed to 37°C, loaded into Cunningham chambers (Cunningham 1965), sealed with molten wax and incubated at 37°C. Plaques are visible to the naked eye, and counted after 45 minutes by a stereomicroscope. The number of PFC was

expressed as PFC/10^6 viable spleen cells.

3.2.7 Delayed type hypersensitivity / insulin treatment regimen

In the present study DH was measured using the ear swelling assay (Phanuphak et al 1974). Sensitisation was performed by applying 25 µl of 0.5% DNFB, w/v, in a solution containing 4:1 acetone and olive oil, onto the shaved flank skin of mice. After one week, the mice to be challenged were lightly anaesthetised with ether and 10 µl of 0.2% DNFB was applied to the dorsal surface of the right ear. The subsequent ear swelling was measured, 24 hours after the secondary challenge, with an engineers dial gauge (Gauge Service and Supply Co. Ltd., UK).

Following sensitisation on days 10, 20 or 70 and secondary challenge with DNFB one week later, DH was measured in 3 separate groups, as shown in Table 3.1. In one group, 5 MSZ treated mice received insulin injections 1.5U subcutaneously, twice daily, from experimental day 10 until the end of the study (Insulin B.P. prepared from ox pancreas was from The Boots Company, PLC, UK) . As a control another 5 MSZ treated mice received saline injections instead of insulin. Kinetic studies of DH response were carried out on one group of mice, as shown in Table 3.1; in this group ear thickness measurements were made 24, 48 and 72 hours after the secondary challenge with DNFB. After the last measurement the spleen and thymus gland were removed and weighed.

DH was assessed by an ear enlargement index:

$$\text{ear englargement index} = \frac{\text{Thickness of right ear pinna (experimental)}}{\text{Thickness of left ear pinna (control)}}$$

Table 3.1 Protocol for DH Studies

Experimental day	day 10 group	day 20 group	day 70 group
0	control 1st SZ injection	control 1st SZ injection 1st SZ injection	control 1st SZ injection
10	1° DNFB sensitised	saline injection insulin injection	
17	2° DNFB challenge		
18	DH Measurement		
20		1° DNFB sensitised	
27		2° DNFB challenge	
28		DH measurement (24 h)	
29		DH measurement (48 h)	
30		DH measurement and lymphoid organ weight measurement (72 h)	
70			1° DNFB sensitised
77			2° DNFB challenge
78			DH measurement

3.3 RESULTS

3.3.1 Spleen and thymus weight and cellularity

Both spleen and thymus weights were significantly reduced ($\rho < 0.05$) in TO mice which received SSZ injections (Table 3.2). The reduction in lymphoid organ weights was also reflected in the lymphocyte numbers. The thymus weight, in particular, was reduced following SSZ injection, representing only $36 \pm 7\%$ of the weight of the thymus in control mice. Spleen weights were also reduced significantly following SSZ treatment. In contrast however MSZ treatment did not produce any atrophy of the lymphoid organ weights.

3.3.2 Spleen Weight Following Immunisation with SRBC

Table 3.3 demonstrates that the spleen weight was significantly raised following SRBC injection in both MSZ treated and control mice. Following SRBC immunisation the increase in the size of the spleen in control mice was greater than the increase in the MSZ treated mice.

3.3.3 Anti - SRBC HA titres

HA titres against SRBC, measured 7 days after immunisation, are shown in Figure 3.1. MSZ treated mice ($-\log_2 5.8 \pm 0.9$) had significantly depressed antibody titres when compared to the control levels ($-\log_2 7.9 \pm 0.6$).

3.3.4 Antigen Reactive Cells (ARC)

Figure 3.2 depicts the number of ARC measured between 1-15 days post SRBC

injection. Numbers of ARC in the spleen of MSZ treated mice were significantly lower 7 and 9 days post SRBC injection when compared to the non diabetic controls. The peak of the ARC in the spleen of the diabetic mice was diminished but not delayed.

3.3.5 PFC assay

Figure 3.3 shows that the numbers of PFC against SRBC were decreased in the spleen of MSZ treated mice when compared to controls. The anti-SRBC PFC response in MSZ treated mice was not delayed and peak numbers of PFC were detected 4 days after the SRBC injection in both control and diabetic animals.

3.3.6 Delayed Hypersensitivity

Figure 3.4 shows that, when measured 20 and 70 days after the 1st SZ injection, the DH response was found to be depressed in MSZ treated mice when compared to control mice. However the DH response was not significantly lowered when measured 10 days after MSZ treatment. Kinetic studies of the DH reaction (Figure 3.5) indicate that DH was depressed and not merely delayed in the diabetic mice. DH in MSZ treated mice was significantly depressed 24 hours following the secondary challenge of DNFB. The increase in spleen and thymus weights observed in control mice was significantly less in MSZ treated mice after sensitisation and challenge with DNFB (Table 3.4). Daily insulin treatment of MSZ treated mice restored the DH response to a level which was not significantly different from the control. Insulin therapy also restored the weight of the thymus in diabetic mice to a level comparable with those found in control mice. In contrast, the spleen weight was not completely restored in MSZ mice following insulin treatment.

Table 3.2 Effect of SZ treatment on lymphoid organ weight and cellularity

	Control n = 5	SSZ n = 5
Thymus wt (mg)	28 ± 2	10 ± 2 * (36 ± 7%)
Thymus cellularity X 10^7	5.7 ± 1.5	0.17 ± 0.03 *
Spleen wt (mg)	116 ± 15	57 ± 11* (49 ± 9%)
Spleen cellularity X 10^8	2.1 ± 0.5	0.6 ± 0.1 *

Measurements were made 13 days after SZ. Figures in parenthesis indicate % values of the control, * $p < 0.05$ compared with control.

Table 3.3 Effect of SRBC immunisation on spleen weight in MSZ treated mice

	Control	MSZ
Spleen wt (mg) before immunisation on experimental day 10 (n = 5)	118 ± 27	104 ± 7
Spleen wt (mg) after immunisation with SRBC (n = 8)	151 ± 8	126 ± 9 *

Spleen weight before immunisation was measured on day 10 after the 1st SZ injection. SRBC were injected on day 10 and spleen weights were measured again 7 days post SRBC injection, * $p < 0.05$ compared with control.

Table 3.4 Effect of DH to DNFB on lymphoid organ weight

	MSZ n = 5	MSZ + insulin n = 5
Thymus wt % of control †	48 ± 18 *	105 ± 10
Spleen wt % of control †	66 ± 14 *	70 ± 5 *

Measurements and insulin treatment were as indicated in Table 3.1.

† denotes control mice which did not receive MSZ-treatment but were treated with DNFB

* $p < 0.05$ compared with control

FIGURE 3.1

SERUM ANTIBODY TITRES TO SRBC IN CONTROL AND MULTIPLE-STREPTOZOTOCIN TREATED (MSZ) TO MICE. FIGURES ARE EXPRESSED AS MEAN ± SEM WITH THE NUMBER OF DETERMINATIONS IN PARENTHESES. *$P<0.05$ COMPARED WITH CONTROL.

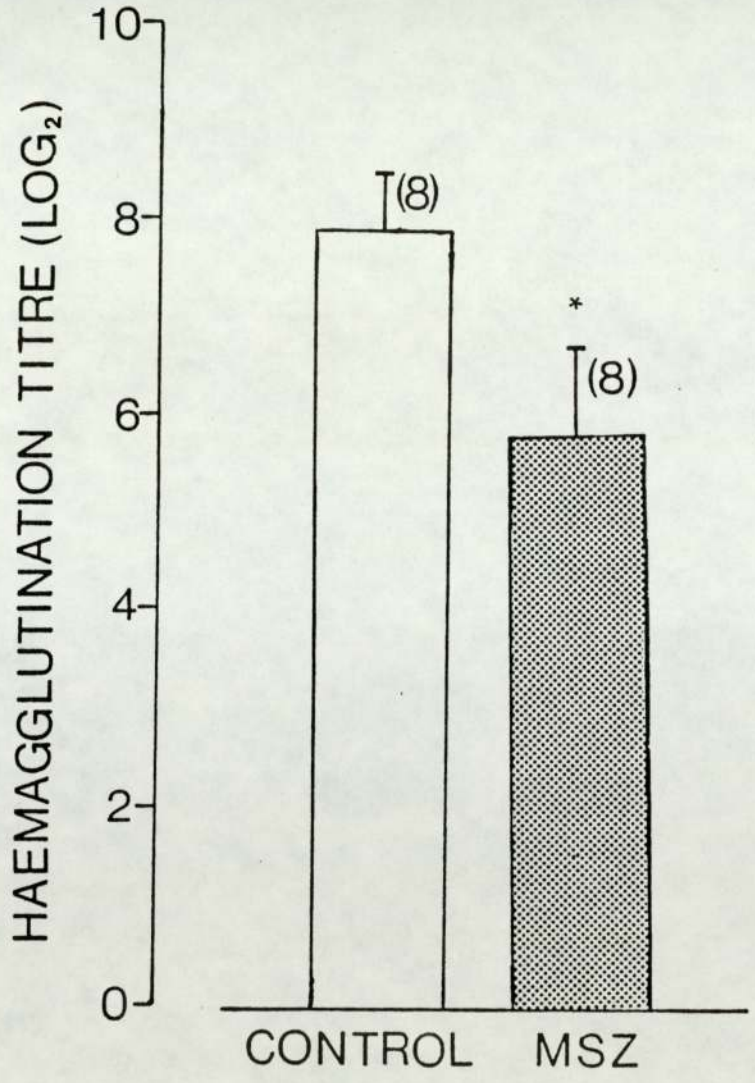


FIGURE 3.2 TIME COURSE OF ANTIGEN RECOGNISING CELLS (ARC) IN THE SPLEENS OF MSZ-DIABETIC AND CONTROL MICE FOLLOWING SRBC CHALLENGE. * $P<0.05$.

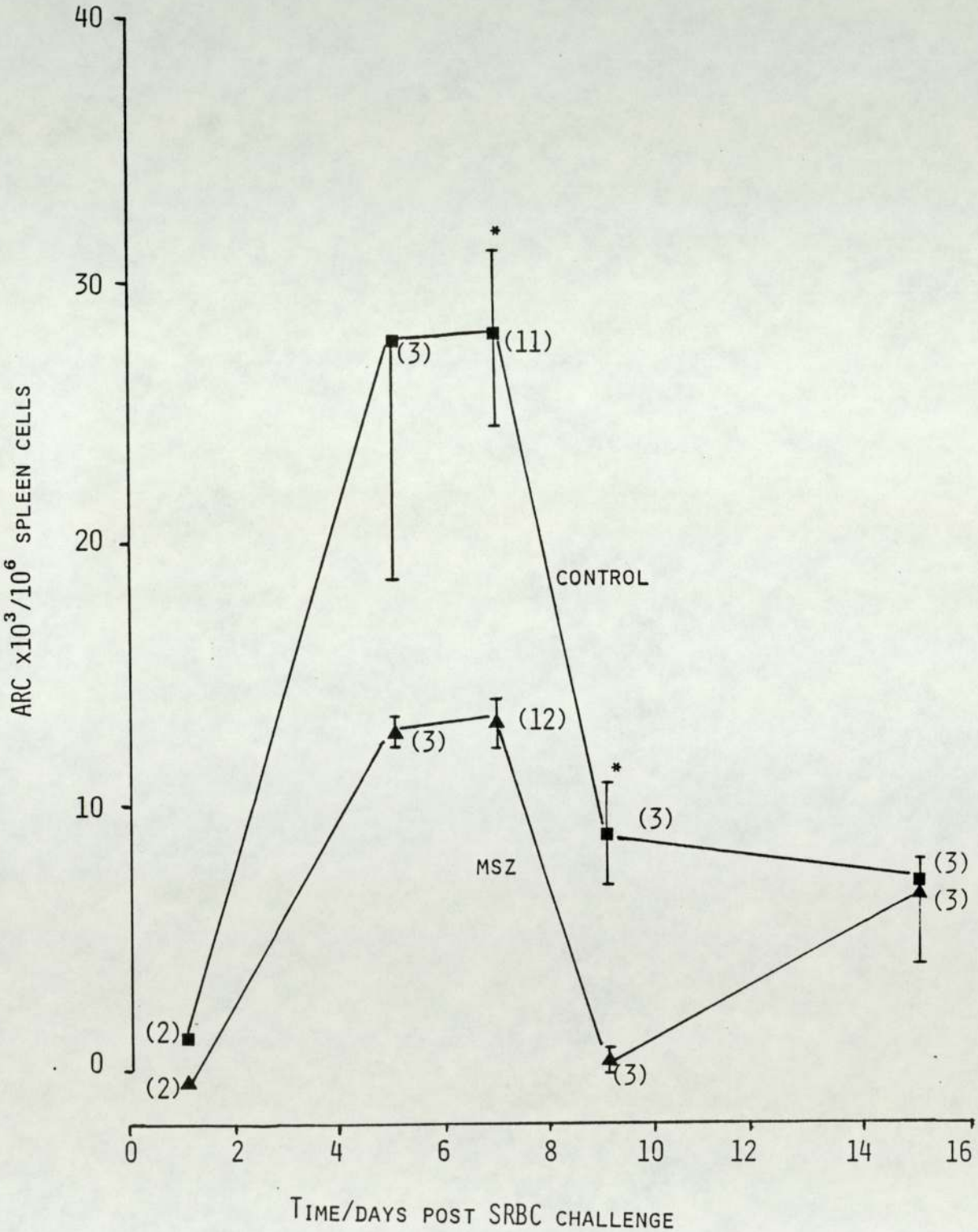


FIGURE 3.3 TIME COURSE OF PLAQUE FORMING CELLS (PFC) IN THE SPLEENS OF MSZ-DIABETIC AND CONTROL MICE FOLLOWING SRBC CHALLENGE * $p<0.05$.

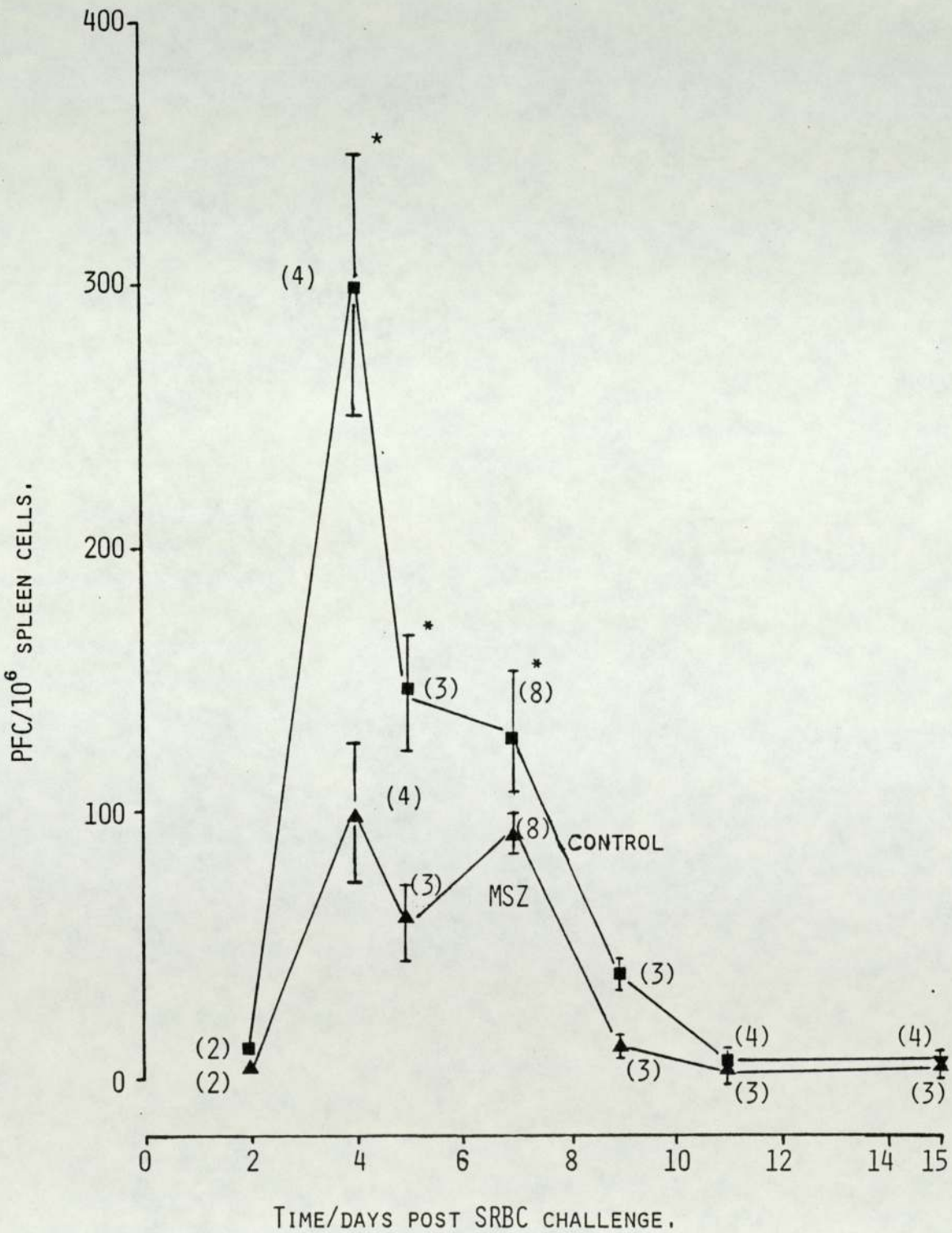


FIGURE 3.4

TIME COURSE STUDY OF DH RESPONCE TO DNFB AND THE EFFECT OF INSULIN TREATMENT IN MSZ-TREATED MICE, FIGURES ARE EXPRESSED AS MEAN ± SEM, FOR PROTOCOL OF DH STUDIES SEE TABLE 3.1
$p^* < 0.05$, N=5.

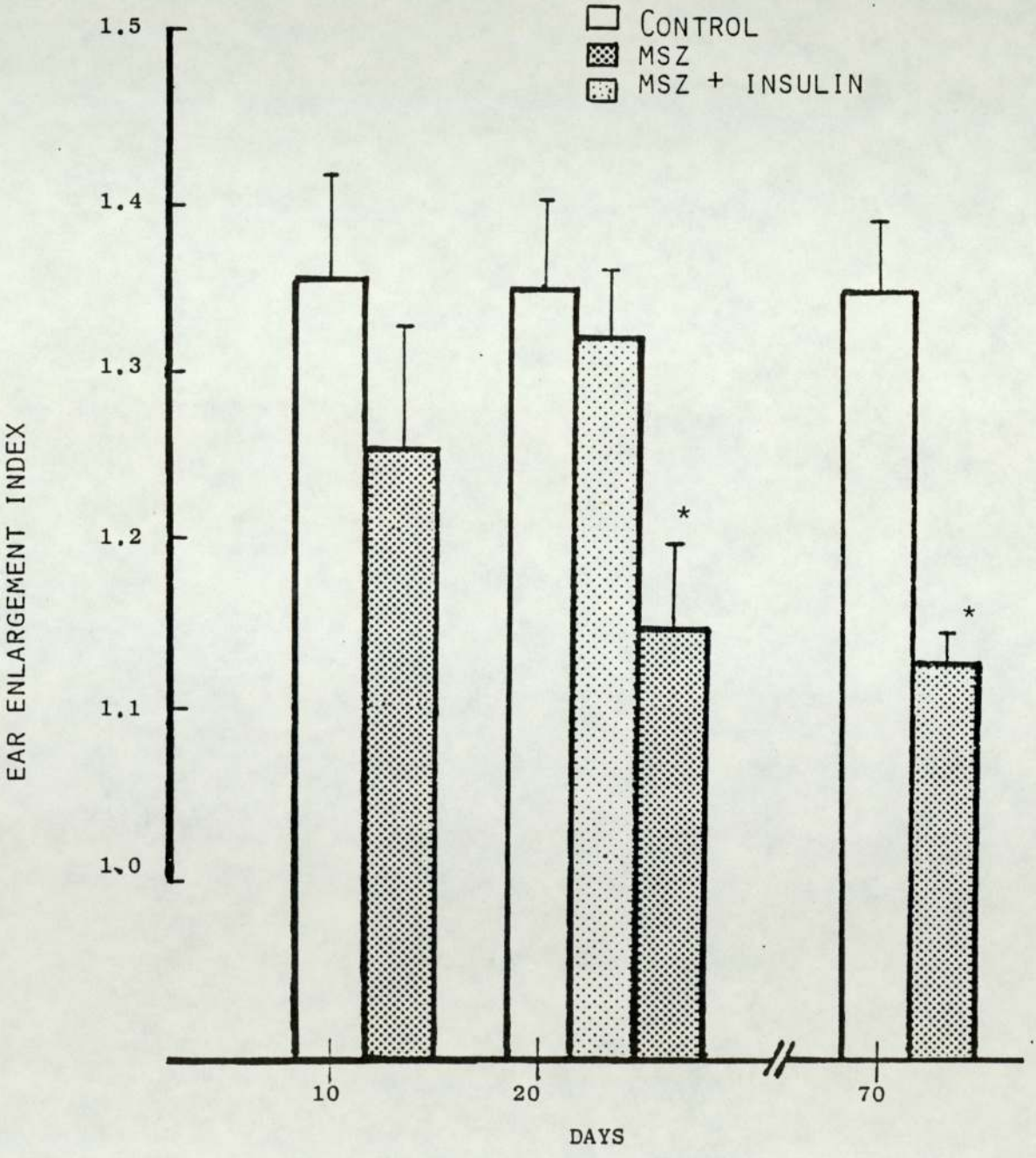
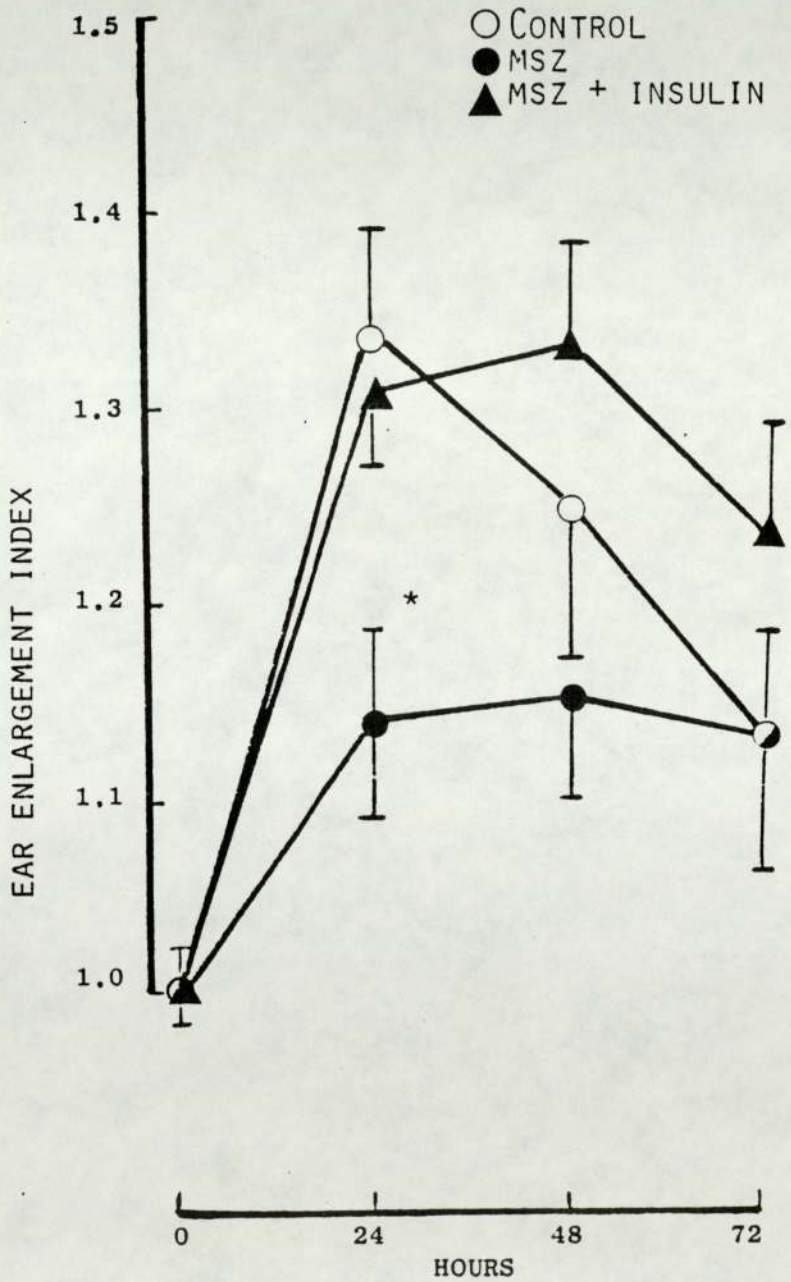


FIGURE 3.5

KINETICS OF DH RESPONSE TO DNFB IN MSZ-TREATED, MSZ PLUS INSULIN TREATED AND CONTROL MICE MEASURED 20 DAYS AFTER THE 1ST SZ INJECTION (SEE TABLE 3.1) *p<0.05, N=5.



3.4 DISCUSSION

Examination of the spleen and thymus after SSZ injection showed that these organs were considerably smaller, by weight as well as cellularity, compared to organs from control mice. SSZ treatment has previously been reported to cause atrophy of the lymphoid organs (Nevalainen and Hoftiezer 1977; Nichols et al 1979 and 1981; Ishibishi et al 1980). Similar findings have also been observed after alloxan induced diabetes in mice (Pavelic et al 1978; Pasko et al 1981). By contrast however, MSZ treatment did not cause atrophy of the lymphoid organs.

In the diabetic state a series of mechanisms may lead to the atrophy of lymphoid organs. One explanation put forward was that atrophy may be due to the stress experienced by diabetic mice. In diabetes, cortisone and hydrocortisone levels are increased, and administration of these adrenal hormones in mice produces atrophy in both thymus and spleen. However, the finding that adrenalectomy did not prevent SSZ induced atrophy of lymphoid organs does not support this proposal (Tulsiiani and Touster 1981).

The lack of or depressed levels of insulin, could also depress the normal function of lymphocytes. Insulin facilitates the uptake of glucose into tissues (e.g. liver, muscle and adipose tissue). Similarly, insulin facilitates transport of glucose into lymphoid cells. In the absence of insulin the lymphocyte is unable to transport the glucose necessary for glycolysis. Therefore, even though the concentration of glucose in the plasma of diabetics is higher than in non-diabetics, lymphocytes from diabetics are unable to grow and function properly through lack of insulin.. In addition, it has been

reported that certain enzymes necessary for producing nicotinamide adenine dinucleotide phosphate (NADPH) and ribosides for RNA and DNA synthesis are decreased in patients with diabetes (Brody and Merlie 1970). Probably the best evidence that insulin deficiency can depress the normal growth of lymphoid organs comes from restoration studies. Insulin therapy in SSZ induced diabetic animals, partially restored the lymphoid organ cellularity (Nichols et al 1979 and 1981). Furthermore, insulin therapy also restored lymphoid cellularity in alloxan induced diabetic mice (Mahmoud et al 1975; Pavelic et al 1978). Further support for the role of insulin in normal growth of lymphoid organs comes from experiments in which the possibility of direct cytotoxicity of lymphocytes by SZ is eliminated. In pancreatectomized rats, the thymus was reduced in weight and cellularity, splenic size was normal, but there was a profound depletion of small lymphocytes in the perifollicular mantle (Fabris and Piantanelli 1977).

In an attempt to further examine the role of insulin on lymphoid organ weight, spleen weights in ob/ob, mice were investigated. Preliminary studies suggest that spleen weights in the ob/ob mice were significantly lower when compared to the sex and age matched HO mice. This finding was in agreement with previous work on C57BL/6J ob/ob mice (Meade et al 1979), and on db/db obese mice (Fernandes et al 1978). But, since ob/ob mice had elevated levels of insulin, it is provocative to speculate that decreased lymphoid organ weight is always associated with depressed insulin levels. It is likely that depressed lymphoid weights in ob/ob mice are most likely due to some other mechanism.

Although it is tempting to attribute the atrophy of lymphoid organs to deficiency of insulin in MSZ treated mice, streptozotocin might also have a direct injurious effect on the cells of lymphoid organs (Nevalainen and Hoftiezer 1977; Nichols et al 1979 and 1981). in-vitro experiments have shown that SZ decreases the viability of spleen and thymus cells (Gaulton et al 1985). Wellhausen et al (1986) found that, in-vivo, lymphoid cytotoxicity preceded the onset of hyperglycaemia in SZ (50 mg/kg body weight) treated mice. The authors demonstrated that SZ treatment depleted thymocytes and depressed the lymphocyte/neutrophil ratio. Whilst a lower concentration of SZ was used, in the present studies, it is possible that even the 40 mg/kg SZ injection used here may also be cytotoxic, especially when 5 injections are administered. It was important therefore, to investigate whether the MSZ treatment had any cytotoxic effects on lymphocytes.

MSZ treatment did not cause atrophy or a reduction in spleen weight in non-immunised animals in the work presented here. However, the gain in the spleen weight which normally occurs following antigenic challenge was significantly less following SRBC immunisation in MSZ treated mice compared to the gain seen in immunised non-diabetic control mice. This result is similar to those observed in SRBC immunised alloxan treated mice (Pavelic et al 1978). However, it is important to note that in alloxan diabetic mice, the spleens were initially smaller than the spleen in the control mice. At the levels used in the present study it may be that MSZ treatment only affects antigen driven lymphocyte proliferation and does not affect the normal proliferation processes seen during development of lymphoid organs.

In the present study the specific immune response to a thymus dependent antigen, SRBC, was investigated in MSZ treated and control mice. Following SRBC immunisation, the anti-SRBC HA titre in the peripheral blood was determined and there was a significant suppression of the HA titres in MSZ treated mice. This finding is in agreement with other reports of the affects of MSZ treatment in other strains of mice (Kim and Steinberg 1984; Nakano et al 1984). Nakano et al (1984) induced diabetes using different doses of SZ in mice. Consequently, the mice became diabetic but with different levels of hyperglycaemia. HA titres to SRBC in these mice were depressed and also reflected the level of hyperglycaemia i.e. the greatest depression of HA in mice was found in mice with the highest blood glucose levels. Ludwig et al (1976) reported that agglutinin titres to E.coli and staphylococcal antigens were lower in IDDM, and that the percentage of diabetics which did not have antibodies to pertusis and diptheria toxoid was increased when compared to control levels.

In addition to reduced antibody titres to SRBC, the number of antigen specific lymphocytes in the spleen of SRBC immunised diabetic mice was also depressed as measured by the immunocytoadherence, or antigen reactive cell (ARC) assay. Whilst no previous reports on the effect of MSZ induced diabetes in ARC have been found, Busby et al (1983) reported that ARC were depressed in SSZ treated mice. The depression of ARC in MSZ treated mice, following SRBC immunisation, is in accordance with the depressed HA titres also seen in MSZ treated mice. The kinetics of the ARC following SRBC immunisation was also examined in MSZ treated mice, to determine whether the peak response in MSZ treated mice was merely delayed. There was no delay in the peak ARC response in MSZ treated mice, but levels of ARC were significantly depressed.

The ARC assay used in the present study is perhaps more sensitive than the PFC assay, because it measures both T- and B-lymphocytes with specific receptors for the antigen, whereas the PFC assay measures only mature antibody secreting B-lymphocytes. Since T- and B-lymphocytes are required for an efficient immune response against SRBC, depressed ARC in MSZ treated mice may reflect an overall depression in immune responsiveness.

In the present study, the numbers of anti-SRBC PFC were diminished in the MSZ treated mice. This finding was in agreement with reports on the effects of MSZ treatment in other strains of mice (Nakano et al 1984; Kim and Steinberg 1984). However, Barg et al (1980) found that MSZ treated mice were able to generate PFC responses which were similar to the control values based on the number of cells in the spleen, although the total PFC per spleen was depressed in the MSZ treated mice. PFC responses have also been reported to be depressed in alloxan (Ptak et al 1977; Pavelic et al 1978; Maduna et al 1980; Gaulton et al 1985) and SSZ treated mice (Ishibishi et al 1980; Saiki et al 1980;, Handwerger et al 1984; Gaulton et al 1985).

We also examined the kinetics of the appearance of PFC after SRBC immunisation, to determine if the PFC response in the MSZ treated mice was delayed. There was no delay in the ability of MSZ diabetic mice to generate PFC in-vivo. We were unable to find comparable kinetic studies of MSZ treated mice. However, Busby et al (1983) have reported that the PFC response to pneumococcal polysaccharide SIII (S3) was delayed and the peak response was not suppressed in SSZ treated mice. This may

reflect a defect in the division rate or regulation of the immune response in the diabetic, following antigenic stimulation. However, since S3 is a T-independent antigen the difference in the results cannot be compared directly, since there is no requirement for the T cell help which is necessary for the proper development of antibody responses to SRBC.

In contrast to the situation reported here, PFC have been found to be increased in obese, hyperinsulinaemic diabetic db/db mice (Fernandes et al 1978). It is tempting to speculate that perhaps the depressed PFC response in MSZ treated, alloxan or SSZ treated mice might be due to hypoinsulinaemia. This observation is supported by a number of studies in which depressed PFC responses could be reversed by insulin therapy (Pavelic et al 1978; Slijepcevic et al 1978; Ishibishi et al 1980; Nakano et al 1984; Gaulton et al 1985. See also work in the present chapter on restoration of impaired DH responses). PFC responses to SRBC have been found to be enhanced in NOD mice (Naji and Barker 1984). The reason for these contrasting findings between MSZ treated mice and NOD mice is not clear. The discrepancies suggest that the immunological abnormalities in the MSZ treated mice differ from those in NOD mice in spite of many other similarities, such as insulitis and ICSA. Further work is required to clarify this issue.

The humoral immune response against SRBC requires the collaboration of T-helper cells prior to the formation of antibody. But the ability of the antibody forming B cell can be assessed independently of the T-helper cell by using T-independent antigens. Saiki et al (1980) reported that the PFC response to TNP-LPS, a T-independent

antigen, was intact in SSZ treated mice. These results suggest that at least some B lymphocyte responses are normal in SSZ treated mice. It is possible that the depressed PFC formation against T-dependent antigens is due to the abnormal function of T-helper cells. By way of contrast, Busby et al (1983) found that PFC responses to S3, a T-independent antigen, were depressed in SSZ treated mice. Furthermore, anti TNP-LPS PFC responses have also been reported to be depressed in alloxan diabetic mice (Ptak et al 1977). Whilst the differences in responses of alloxan and MSZ treated mice to the T-independent antigens may be due to the different diabetogenic drugs used, the nature of the discrepancy within SSZ treated mice is uncertain. The difference in the T-independent antigen responses of MSZ and SSZ treated mice could, however, be due to the toxic effects of SSZ on the lymphocytes (Nichols et al 1978; Gaulton et al 1985).

Delayed hypersensitivity (DH) is a cell mediated response which relies on the interaction of specifically sensitised T cells (T_{DH}), and recruited monocytes (Miller and Jenkins 1986). The present investigation examined delayed (type IV) cell mediated immunity (CMI) by measuring the DH response to DNFB. There was a significant depression of DH in the MSZ treated mice. However, the impairment of DH varied with time after induction of diabetes. Initial measurements, made during the early phase (day 10) after MSZ treatment, indicated that the magnitude of the DH response in MSZ treated mice was not significantly different from that of control mice, even though the MSZ treated mice were overtly hyperglycaemic. Two subsequent measurements, at 20 and 70 days, indicated significantly depressed DH in the MSZ treated mice. This finding is in general agreement with an earlier report on DH responses in MSZ treated mice (Barg et al 1980). These authors, however, treated

their mice with cyclophosphamide, an immunosuppressive drug, which inhibits the action of B-lymphocytes. Since B-lymphocytes produce ICSA and these antibodies have been found in MSZ treated mice (Itoh et al 1984), it is possible therefore, that cyclophosphamide might interfere with the normal progression of diabetes in MSZ treated mice. Consequently, it is difficult to compare the results presented here directly with those of Barg et al (1980), since, even though the above authors found that DH was depressed, the severity of the depression could have been affected by cyclophosphamide.

A delayed, diminished cell mediated immune response was observed in alloxan diabetic mice (Sood et al 1975). Similarly, Ishibishi et al (1980) reported that DH was delayed in SSZ treated mice. In the present study, kinetic studies of the DH response demonstrated that maximal values were depressed in MSZ treated mice but were comparable to normal values by 72 hours. The reduced DH response was also consistent with findings for the PFC response and the ARC response against SRBC in MSZ treated mice.

Following sensitisation and challenge with DNFB, the size of both spleen and thymus in MSZ treated mice were significantly depressed compared to those of controls, however, the weights of lymphoid organs prior to sensitisation with DNFB in MSZ treated mice were comparable to the controls. These findings are consistent with findings presented here on lymphoid organ size following SRBC challenge. Ptak et al (1977) has reported that alloxan diabetic mice also had smaller spleens and thymus compared to control mice following challenge with oxazolone (a contact sensitiser).

Roth et al (1980) observed that spleen weights were depressed in alloxan treated mice following challenge, but not sensitisation with DNFB. Similar findings have been observed in DH transfer studies in both MSZ treated mice (Barg et al 1980) and in alloxan treated mice (Roth et al 1980). Roth et al found that DH could equally well be transferred by spleen cells from sensitised normal or alloxan diabetic mice into normal mice, but that DH was attenuated when spleen cells from sensitised normal or alloxan diabetic mice were transferred into diabetic mice.

Insulin therapy partially restored the weight of the MSZ treated spleen and completely restored the thymus weight to that observed in non-diabetic DNFB sensitised mice. Furthermore, insulin therapy completely restored the levels of the DH response in MSZ treated mice to that observed in control mice. Similar results have been found in alloxan treated mice (Ptak et al 1975; Roth et al 1980), and in SSZ treated mice (Gaulton et al 1985). In support of the role of insulin on CMI, Arquilla et al (1980) reported that DH was reduced when normal mice were made insulinopenic after the injection of insulin antisera. There have also been reports which found that the depressed anti-SRBC PFC responses in alloxan treated mice (Ptak et al 1977; Pavelic et al 1978; Gaulton et al 1985) and in SSZ treated mice (Ishibishi et al 1980; Gaulton et al 1985) could be reversed by insulin therapy. If it can be said that the depressed immunological function is singularly due to insulinopenia, then insulin therapy should completely restore immunological function. However, since the restoration of immune responses is often only partial (for review see Drell and Notkins 1987); it could be that other islet hormones are also required for the normal function of immune responses. Islet transplantation into SSZ treated mice has been reported to completely reverse the

depressed immune function (Handwerger et al 1984; Hadzija et al 1984). In the present study glocose/insulin levels were not measured following insulin therapy and so it is unclear what role insulin may have played in restoring immune function.

Whilst immune responses of diabetic mice to some antigens are depressed, others are normal. In general, observations seem to indicate that immune responses to T-dependent antigens are depressed whilst immune responses to T-independent antigens are less commonly affected. One possible explanation of this could be that insulinopenia has an unequal effect on different subpopulations of lymphocytes. Krug et al (1972) and Helderman and Strom (1978) have reported that insulin receptors are present on activated T-cells but not on resting T-cells. The identification of subpopulations of lymphocytes, which may be affected by insulinopenia, awaits attention. For instance, it would be of interest to examine whether T_H cells were specially affected by insulinopenia. If this were so, then it may explain why T-dependent immune responses are diminished in diabetic animals. The use of monoclonal antibodies specific for cellular subsets of T lymphocytes would be useful in clarifying this point. The exact role of insulin in immune function is not known, but presumably, saturation of specific insulin receptors on lymphocytes, by insulin, results in an increased accumulation of intracellular cyclic guanosine monophosphate (GMP), (Strom et al 1975). The role of cyclic GMP is important in immune responses because it augments lymphocyte proliferation, antibody production (Watson. 1975), and T-lymphocyte cytotoxicity (Strom et al 1973).

Whilst it is difficult to argue conclusively that altered immune funtion in MSZ induced

diabetes is a consequence of the diabetic state and not due to the direct cytotoxicity of SZ on lymphocytes, several aspects of the present study are worthy of note. Firstly, the size and cellularity of spleen and thymus organs were unaffected following MSZ treatment. Whilst these parameters in isolation are not a reliable guide to the lack of possible cytotoxic effects of SZ, the fact that insulin treatment restored the DH response to normal is a strong argument for the persistence of normal cellular function in at least those types of lymphocytes involved in DH reactions. This question is examined further in Chapter 4 where in-vitro mitogen induced blastogenesis is examined following MSZ treatment but prior to the onset of hyperglycaemia.

Further work on the effect of diabetes on immune responses is required, since hypoinsulinaemia may have an unequal affect on different lymphocyte subsets. By examining the role of insulin on isolated lymphocyte subsets, we will be able to better understand immune responses in diabetes. If a lack of direct lymphoid cytotoxicity can be established then the MSZ induced diabetic mouse may provide a useful experimental model because, unlike alloxan and SSZ treated mice, where a direct cytotoxicity on lymphocytes has been reported.

CHAPTER 4 LYMPHOCYTE RESPONSES TO POLYCLONAL B AND T CELL ACTIVATORS

4.1 INTRODUCTION

Polyclonal activators are widely used as agents to activate T and B lymphocytes. Since the cellular differentiation events following activation by mitogens are similar to those observed after antigen specific activation, immunologists have used polyclonal activators in many in-vitro experimental models to study lymphocyte function in-vivo.

Phytohaemagglutinin (PHA) is a plant lectin which induces proliferation of T-lymphocytes. It has long been recognised that PHA induced lymphocyte proliferation is diminished in diabetics when compared to normal patients (Brody and Merlie 1970; Delespese et al 1974; MacCuish et al 1974c). Recently, however, lymphocyte responses to PHA have been reported to be intact in Type I diabetics (Ragab et al 1972; Selam et al 1979; Crosti et al 1986). These apparently contradictory findings may be reconciled in part by examining the severity of the disease in the patients examined. The patients of Brody and Merlie had persistent glycosuria and hyperglycaemia. i.e. "poorly-controlled" diabetics, whereas Ragab and his associates used lymphocytes from diabetic outpatients whose diabetes was "well controlled". It appears therefore, that PHA induced proliferation of lymphocytes from poorly controlled diabetics is diminished, whilst lymphocytes from well controlled diabetics have intact responses to PHA. Concanavalin A (ConA) induced proliferation has also

been reported to be depressed in poorly controlled Type I diabetics when compared to well controlled patients, (Selam et al 1979). Similarly, Plouffe et al (1980) and Crosti et al (1986) reported that ConA induced proliferation of lymphocytes from poorly controlled Type I diabetics was depressed when compared to healthy control subjects.

Lymphocyte proliferation induced by pokeweed mitogen (PWM) has also been examined in diabetics. Selam et al (1980) reported that both poorly- and well-controlled Type-I-diabetics had normal proliferation responses. By way of contrast, Itoh et al (1984) reported suppressed lymphocyte proliferation in Type I diabetics when compared to healthy subjects. The reason for the contrasting results are not clear, one explanation might be that the levels of hyperglycaemia were different in diabetic patients used by different researchers.

Mitogen induced proliferation in SSZ treated mice have also produced conflicting results. Brown et al (1977) reported that ConA induced proliferation was depressed but did not give the time of examination after injection of SZ. Later Nichols et al (1978) reported that 8 or 14 days after SSZ treatment, ConA induced proliferation was normal, but when measured on day 20 was depressed. The same authors in 1979 reported that ConA induced lymphocyte responses in SSZ treated mice were normal when measured after 22 or 48 days. More recently, there has been a report that whilst ConA induced proliferation in SSZ treated rats was depressed, PHA induced proliferation was intact (Chi et al 1982).

Clearly, it can be seen that mitogen induced lymphocyte proliferation in IDDM and

SSZ induced diabetes has produced conflicting results. Much work is needed to clarify whether diabetes has any significant effects on mitogen induced proliferation. The purpose of the present study was to study the effects of MSZ induced diabetes on mitogen induced proliferation of T and B lymphocytes. PHA and ConA were used to study T dependent responses and LPS for B-lymphocyte responses. A temporal study was also carried out to investigate how the induction and progression of hyperglycaemia affected mitogen induced lymphocyte proliferation.

4.2 MATERIALS AND METHODS

Mitogen induced lymphocyte proliferation:

A convenient method for quantitating cellular proliferation is to assay DNA synthesis. Incorporation of exogenous radio labelled ^{3}H-thymidine into the cells is measured by scintillation counting. ^{3}H-thymidine, with low specific activity, is used both to prevent radiation damage and to ensure that sufficient exogenous thymidine is available to maintain the incorporation throughout the labelling period. Pulse periods (labelling period) of 4 to 6 hours are sufficient to demonstrate significant incorporation. The magnitude of the response will depend upon the cells used as well as cell density, concentration of mitogen and culture conditions.

In the present study, T cell mitogens ConA and PHA, and B cell mitogens LPS were used to determine the proliferative responses of the spleen cells from MSZ treated and control mice, as described in Mishell and Shiigi 1980. Sterile technique was used throughout, in order to minimise bacterial contamination which can distort the results

by causing excess label to be incorporated into the DNA of replicating bacteria or by interfering with the response of lymphoid cells

A preliminary study was undertaken to determine the time period required to produce the peak proliferative response. Cells were incubated in the presence of ConA at 5 μg/ml for culture times of 24, 48 and 72 hours (Table 4.1). Maximum proliferation was observed after 48 hours of culture. This time period was also adopted for the subsequent investigations with PHA and LPS. The concentration of mitogen required to generate an optimal proliferative response was also determined (Tables 4.2, 4.3 and Figure 4.5).

Triplicate cultures were established in 96-well microtitre plates (Flow Laboratories, UK), containing complete culture medium prepared as described in Chapter 2. A total of $5x10^5$ splenocytes per well were cultured with appropriate mitogen concentrations. All mitogens were purchased from Sigma Chemical Company, UK. PHA was employed at final concentration of 8 μg/ml, ConA at 5 μg/ml and LPS at 50 μg/ml. Cultures were incubated at 37°C in a 5% CO_2 humidified atmosphere for 48 hrs. During the last 4 hrs of culture, cells were pulsed-labelled with 1 μCi of ^{3}H-methyl-thymidine (specific activity 2 Ci/mMol; Amersham Ltd., UK). After the pulse period, the cultures were harvested onto glass-fibre paper (Flow Laboratories, UK) with a multiple-sample cell harvester (Flow Laboratories UK). Filter papers were air dried, placed in a scintillation cocktail (prepared as described in Mishell and Shiigi 1980) and counted in a β-counter. (Beckman Instruments Inc. USA). Means of triplicate cultures were calculated and the results expressed as:

$$\text{Stimulation index} = \frac{\text{cpm of Experimental Sample}}{\text{cpm of basal (no mitogen)}}$$

Table 4.1 Effect of culture time on ConA induced lymphocyte proliferation.

n = 5

Time of culture/hr	24	48	72
Stimulation index	7±2	202±40	132±2

Table 4.2 Effect of concentration of ConA on lymphocytes proliferation after 48 hours of culture.

n = 10

Concentration of ConA µg/ml	0.5	2.5	5.0	10.0
Stimulation index	23±6	148±24	225±27	140±28

Table 4.3 Effect of concentration of LPS on lymphocyte proliferation after 48 hours of culture

n = 5

Concentrating of LPS µg/ml	10	50	100	200
Stimulation index	56±15	88±20	48±8	31±4

4.3 RESULTS:

Proliferative responses of spleen cells from MSZ treated mice were generally diminished to both T- and B-cell mitogens when compared to those of spleen cells from age matched control mice (Figures 4.1-4.5). Proliferative responses of spleen cells from MSZ treated mice to PHA, ConA or LPS (Figure 4.1) were not depressed when measured after 8 days (the time indicates the number of days from the start of the first SZ injection). However, MSZ treated mice were not overtly hyperglycaemic at this stage (see below). In contrast, lymphoblastogenesis (LBG) of MSZ treated spleen cells to PHA was consistently depressed ($\rho < 0.05$) when measured on days 15, 23 and 44 (Figure 4.2). LBG of diabetic spleen cells to ConA was similarly depressed except on day 23 (Figure 4.3). The proliferation of diabetic spleen cells in response to the B-cell mitogen LPS did not follow the same trend as that observed for the T-cell dependent mitogens; whilst LBG was significantly depressed when measured on day 15, responses were comparable to those of control cells on days 8, 23 and 44 of the study (Figure 4.4).

In order to determine the effect of mitogen concentration on proliferative responses of diabetic spleen cells, a dose response curve was carried out. Proliferative responses of diabetic spleen cells to PHA were consistently lower from day 15 when compared to the values obtained for control cells (Figure 4.5).

Following MSZ treatment, the diabetic status of individual mice was confirmed by glucose estimation in individual study animals. 8 days after MSZ treatment there was no significant difference in the glucose values between treated and control mice,

however when glucose was measured after 15, 23 and 44 days, glucose values in MSZ treated mice were significantly different 25±3 mMol/l, 29±4 mMol/l and 47±6 mMol/l respectively, whilst glucose values in control mice were unchanged from the initial 9±2 mMol/l value.

Figure 4.1

Mitogen induced lymphocyte proliferation from MSZ (shaded bar) and control mice (open bar) 8 days after the first SZ dose.
* P = NS, n=5

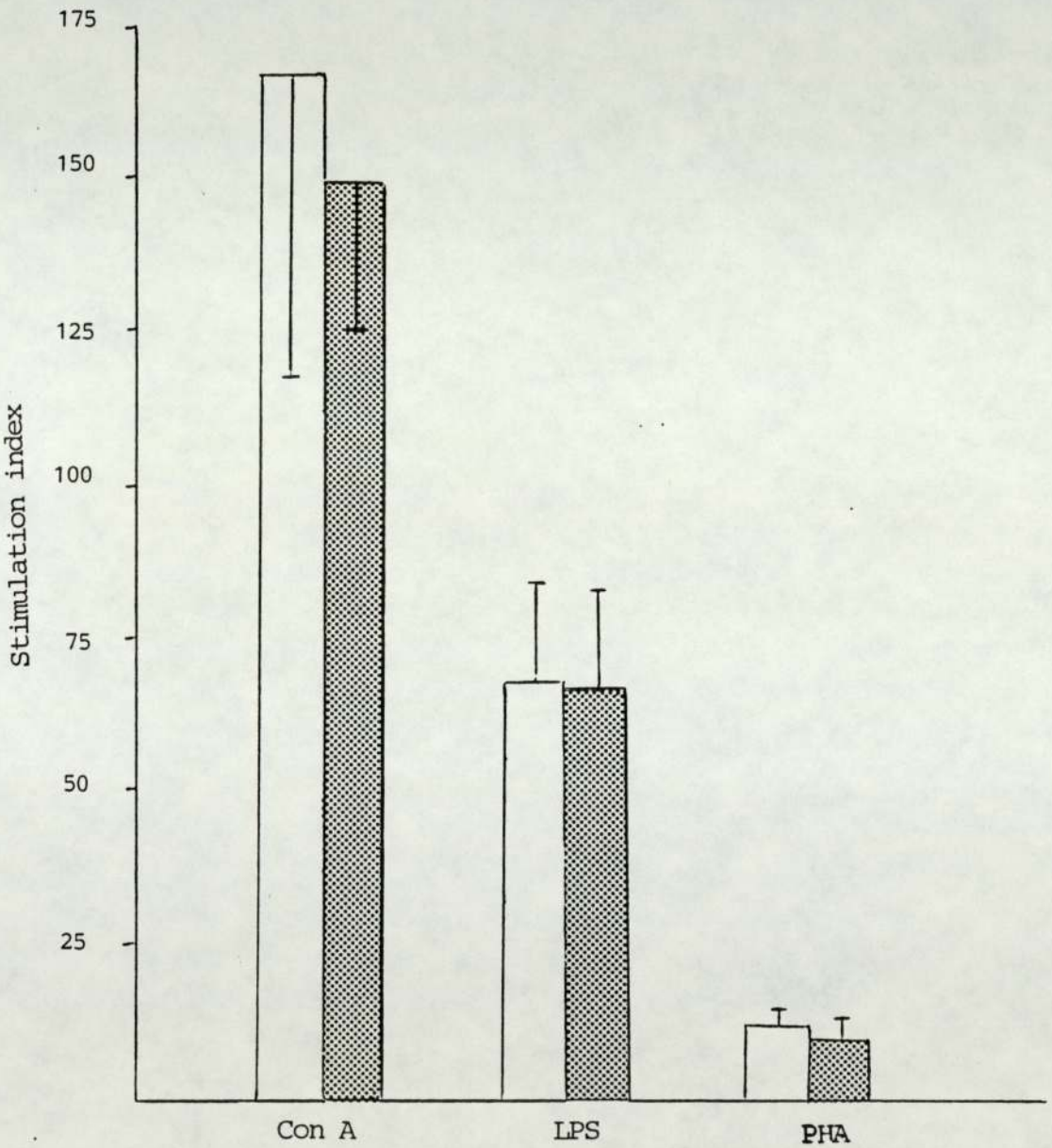


Figure 4.2

PHA induced lymphocyte proliferation from MSZ (shaded bar) and control mice (open bar) * $P < 0.05$, n=5

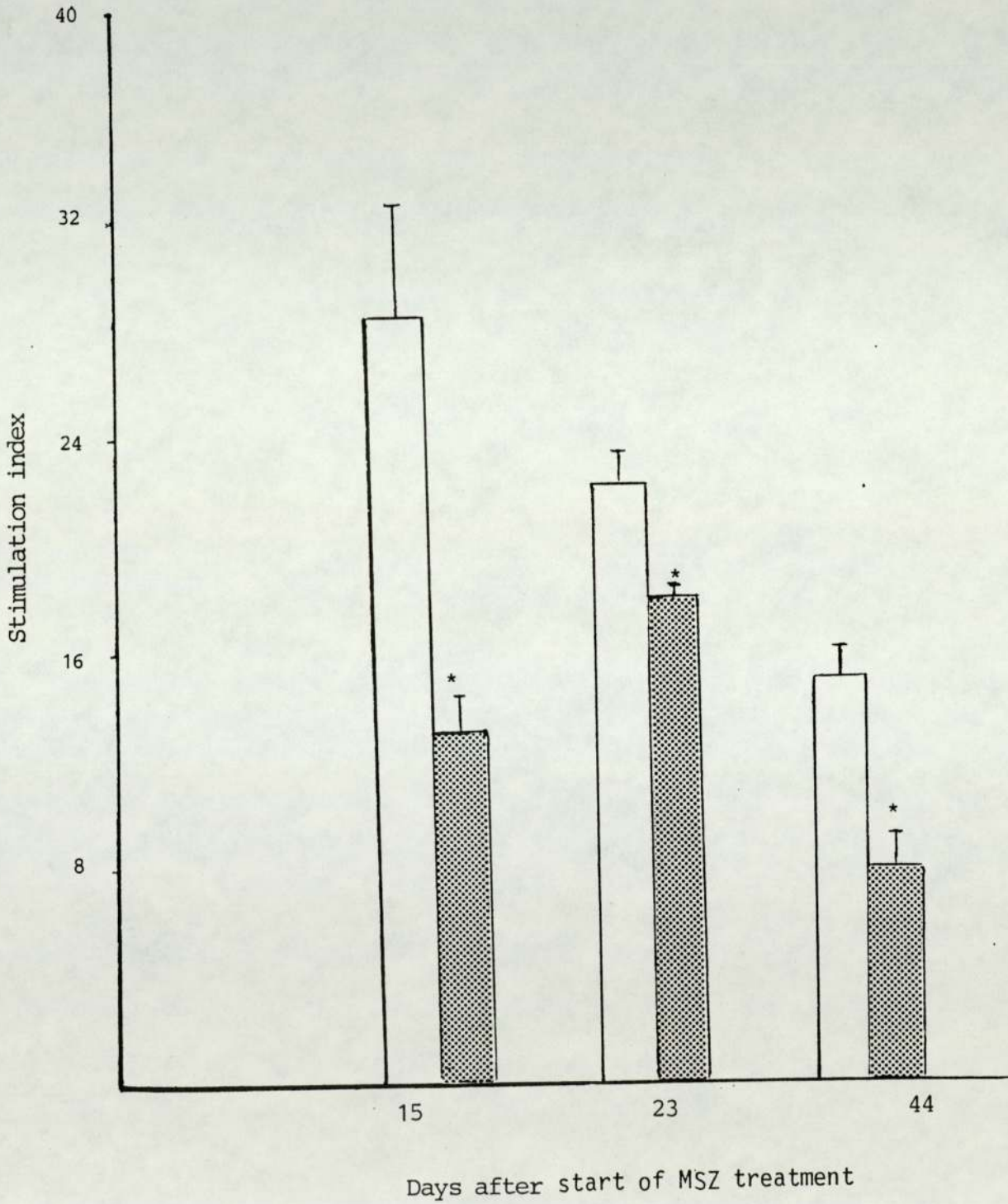


Figure 4.3

Con A induced lymphocyte proliferation from MSZ (shaded bar) and control mice (open bar) * $P < 0.05$, n=5

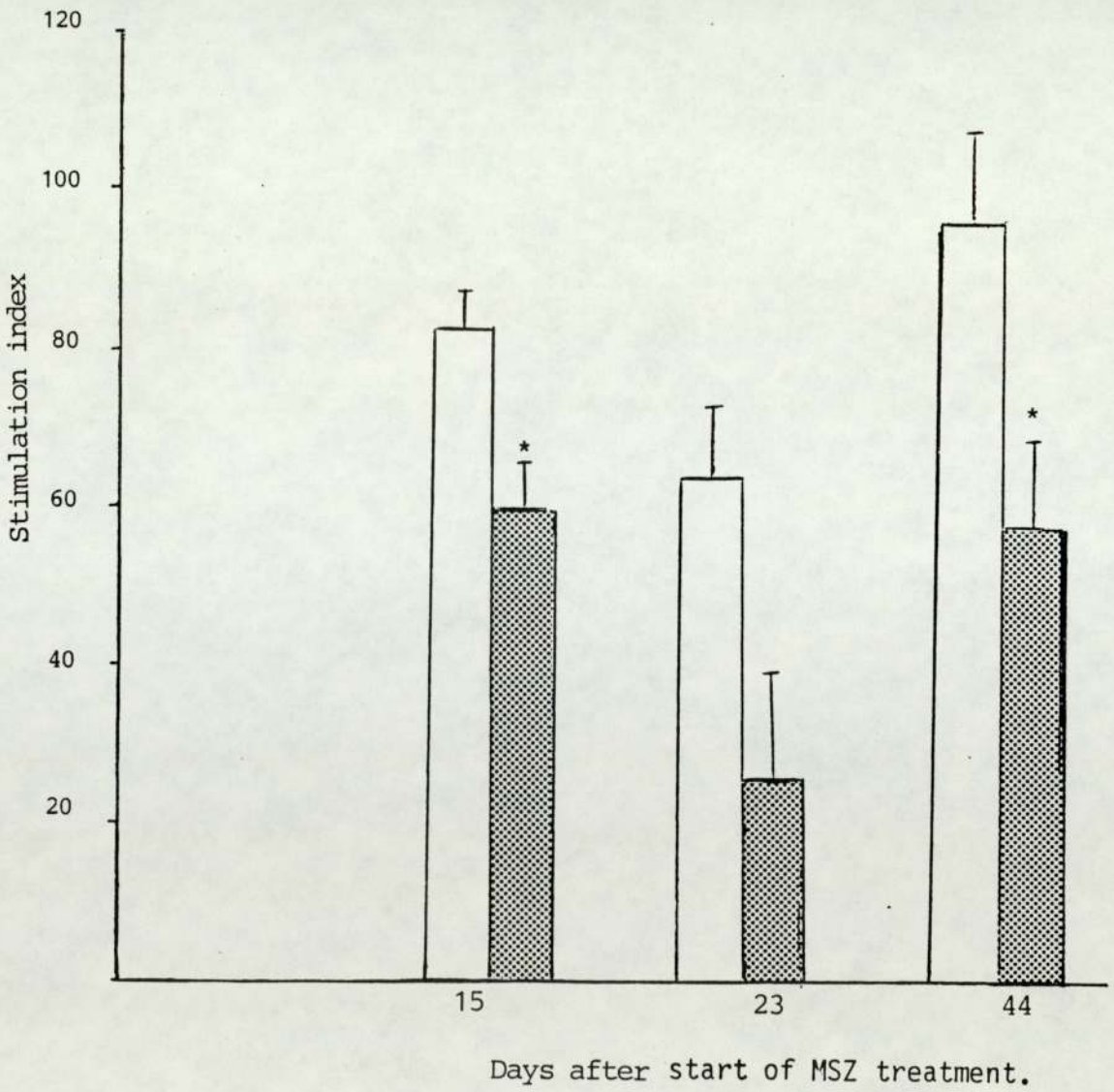


Figure 4.4

LPS induced lymphocyte proliferation from MSZ (shaded bar) and control mice (open bar) * $P < 0.05$, n=5

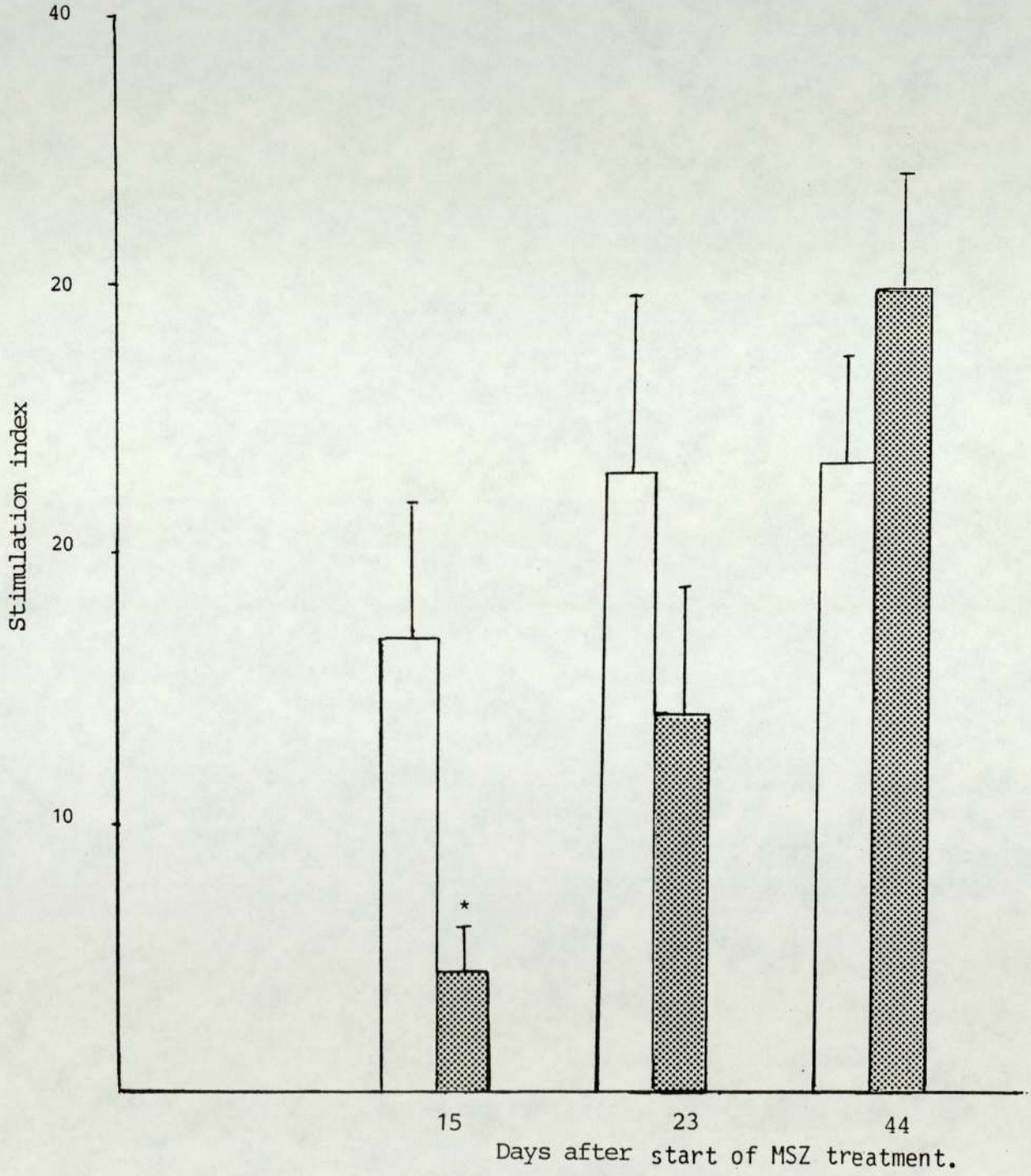
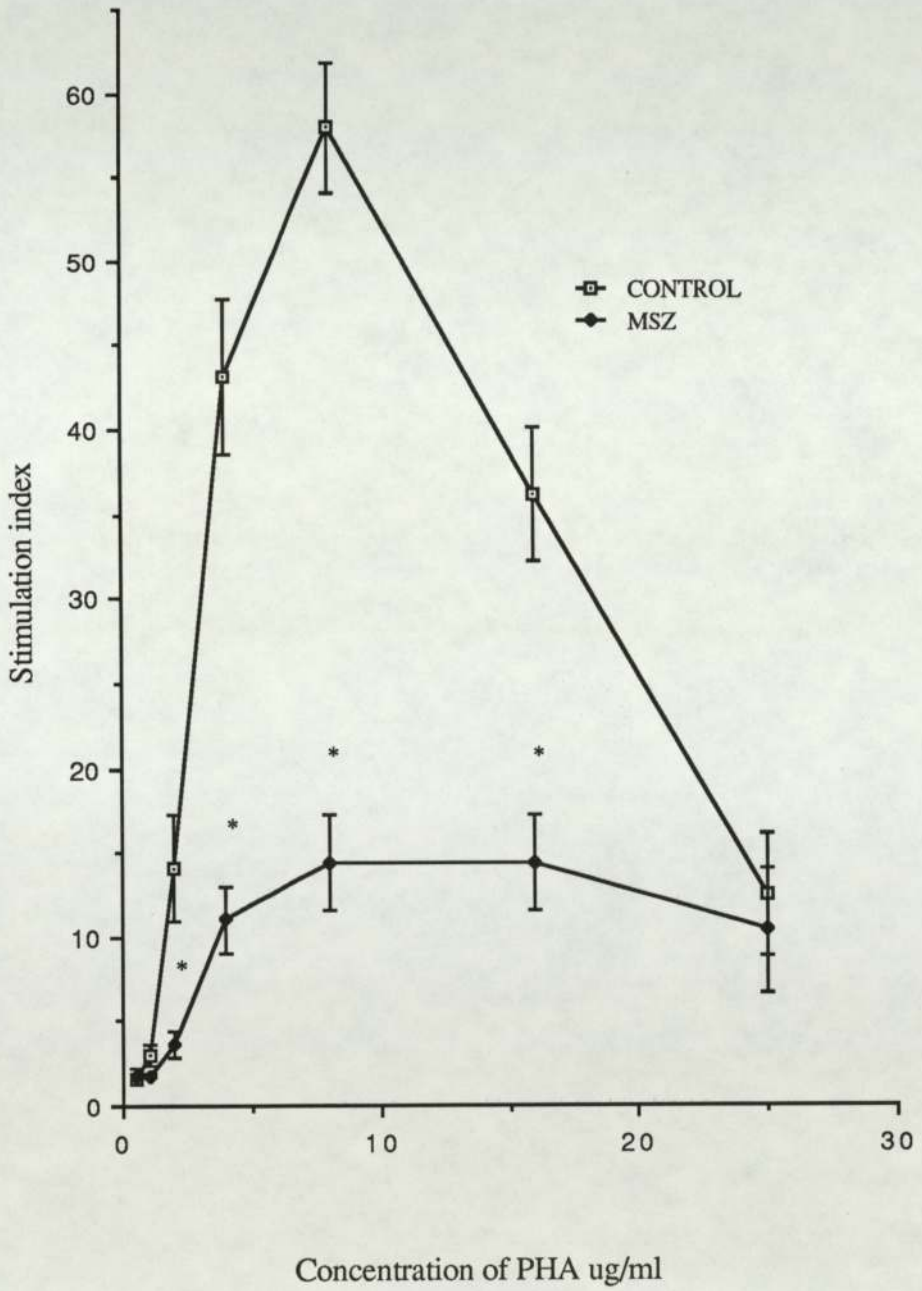


Figure 4.5

Effect of PHA concentration on lymphocyte proliferation from MSZ- treated and control mice, measurements were made 15 days after the first SZ injection

* indicates $p<0.05$, $n = 5$



4.4 DISCUSSION

Proliferation of spleen cells in response to PHA and ConA was significantly depressed in MSZ treated diabetic mice. However, T-lymphocyte responses were intact whilst the mice were not overtly hyperglycaemic. Lymphocyte responses to LPS were generally unaffected in MSZ treated mice, although a significantly depressed response was found on day 15, this depression was transient and the responses on days 8, 23 and 44 were intact.

The results obtained here suggest that mitogen induced T-cell responses are diminished when the mice are also hyperglycaemic. This finding is in general agreement with observations by Leiter et al 1983 and Itoh et al (1984) who also found that LBG to PHA was depressed in spleen cells from diabetic MSZ treated C57BL mice. However, these authors did not detect a depressed response to ConA. The reason for these contrasting findings is not clear, on the one hand it could be a reflection of the different strains of mouse used. In support of this proposal, Itoh et al (1984) found that lymphocyte proliferation of diabetic lymphocytes in response to PHA or ConA was different in the two strains which they used. On the other hand it may be that under certain circumstances one pool or subset of T cells in the spleen is more sensitive to the diabetic state than another subset. It has been found that PHA and ConA stimulate different subsets of T cells (Kimbal 1983). This does not explain however why PHA and ConA responses are diminished in the present study, whilst the lymphocyte proliferation in response to ConA as shown by Itoh et al was still intact.

The depressed LBG response to PHA in MSZ treated mice is probably due to the poor proliferation of the lymphocytes per se and not due to reduced availability of PHA receptor molecules. Increasing the PHA concentration in the culture did not increase the stimulation response of diabetic spleen cells above the control values, indicating that the cells were maximally stimulated at the PHA concentration used.

The fact that LBG was intact before, but not after, the onset of hyperglycaemia suggests that SZ itself is not directly inhibiting cell proliferation, otherwise one would expect that responses would be diminished before the onset of hyperglycaemia. Further studies on the effect of insulin treatment on LBG in MSZ induced diabetic might clarify this, especially if insulin treatment was to reverse the diminished proliferative responses of lymphocytes. Mitogen induced proliferation of spleen cells taken from rats pre-injected with SZ 30 minutes before the assay was performed, have been reported to be normal (Chi et al 1982). In contrast to the results presented here, Itoh et al reported that PHA and ConA induced LBG was defective in mice following treatment with MSZ when assayed 2 days after the start of the MSZ treatment, and before the onset of hyperglycaemia. The reason for these contrasting findings is unclear. Whilst the differences between the results of Chi et al and Itoh et al are obvious, i.e. species specificity, dosage and time of study following SZ treatment, the difference between the results presented here on MSZ treated mice and those of Itoh et al cannot be explained by the above. However differences in the strain of mice might be a possible explanation.

Spleen cells used in the present study contain B and T lymphocytes of differing

subsets as well as macrophages. When considering mitogen induced proliferation it is important therefore, to appreciate that lymphocyte proliferation may be under the influence of other cell populations, either directly (e.g. T-suppressor cells), or via their products (e.g. lymphokines). Indeed it has been shown that lymphocytes from IDDM patients with poor LBG to PHA showed marked improvements in LBG when T-suppressor cells were depleted (Plouffe et al 1979). Similar results were also reported when macrophages were removed from a lymphocyte preparation in the BB rat (Woda and Padden 1986).These studies suggest that the observed depression in mitogen induced proliferation of lymphocytes from IDDM or BB rat could be due to the suppression by suppressor T cells or macrophages.

ConA has been shown to stimulate the secretion of lymphokines from macrophages, which in turn activate other cells. Moreover, T-cell proliferation occurs under the influence of interleukin-1 (IL-1) a macrophage product, and IL-2 a T-cell product (Roitt et al 1985). It might be that the suppression by macrophages and suppressor T cells might cause a defective production of IL-1 and IL-2. Certainly, Gaulton et al (1985) found that leucocytes from SSZ treated mice produced less IL-1 and IL-2 when compared to normal leucocytes cultured in-vitro.

With regard to the suitability of MSZ treated mice as an experimental model for IDDM, it is of interest to note that whilst NOD mice have been considered an excellent experimental model for IDDM, mitogen induced proliferation in the NOD mouse has been reported to be markedly raised (Kataoka et al 1983). Since depressed LBG has been reported in IDDM, BB rat and MSZ treated mice, it is likely that in this respect

NOD mice are not a suitable model to study mitogen responsiveness of lymphocytes in diabetes.

Results presented here suggest that LPS induced proliferation of B-lymphocytes was essentially intact. This finding is in agreement with those of Leiter et al (1983) and Itoh et al (1984), who reported similar results for B cell mitogens. Similarly, proliferative responses of B-lymphocytes to LPS in SSZ treated mice (Brown et al 1977, Handwerger et al 1984) and SSZ treated rats (Chi et al 1982) have been reported to be intact. The normal proliferation of spleen cells in the presence of LPS suggests that the diabetic milieu is only affecting T-lymphocytes. Since in-vivo experiments utilizing MSZ, SSZ or alloxan treated mice have also reported intact humoral immune responses to TI antigens (Chapter 3). It could be that the in-vitro LPS induced proliferation of B-lymphocytes reflects the general B-lymphocyte function in-vivo, however, it is important to realise that mitogens only measure proliferative responses, they say nothing about the general function of the B-lymphocytes.

The exact mechanisms involved in the differential effects of diabetes on T and B-lymphocytes is unclear. In a diabetic state there is (at least) hyperglycaemia and hypoinsulinaemia. Therefore, either of these conditions could influence the mitogenic responses of lymphocytes. Selam et al (1979) reported that lymphocytes from healthy individuals gave poor proliferative responses to the T cell mitogen PHA in the presence of higher than physiological levels of glucose. However, glucose did not have an inhibitory effect on PWM induced proliferation. Furthermore, the same authors reported that following good metabolic control by insulin therapy, PHA

induced lymphocyte proliferation was restored to normal values but ConA and PWM response did not show any distinct improvement. In-vitro studies have demonstrated that insulin enhances the normal proliferative responses of murine T lymphocytes (Snow et al 1983). Pallavicini et al (1976) demonstrated that insulin therapy restored the mitogen induced proliferation of lymphocytes from alloxan induced diabetic mice. Insulin has also been shown to restore wholly or partly a number of other diabetes associated immunological abnormalities (see results presented in Chapter 3 and, for a review, Drell and Notkins 1987). However, these results need to be interpreted carefully since some researchers did not use physiological doses of insulin. Also, since it was demonstrated that insulin therapy only partially restored mitogen responsiveness in SSZ induced diabetic mice (Gaulton et al 1985), this suggests that insulin is not the only factor which can influence lymphocyte activation. It is a possibility that other hormones secreted by the islet may contribute to normal immunological function. Furthermore, it has been shown that whilst insulin therapy only partially restored SRBC induced antibody formation in SSZ treated mice (Ishibashi et al 1980), islet transplantation completely restored it (Handwerger et al 1984).

In conclusion, the work presented here has shown that mitogen induced proliferation of T lymphocytes in MSZ treated mice was depressed, whilst the proliferation of B-lymphocytes was essentially intact. The results also suggest that the defective T lymphocyte responses were due to the diabetic state. The mechanisms involved in the observed abnormalities are not clear, but the restoration of DH responses following insulin treatment suggest that the cause of the depressed proliferative responses observed here are due to insulinopenic effects on T lymphocytes.

CHAPTER 5: NK CELL ACTIVITY AND ADCC IN MSZ TREATED MICE

5.1 INTRODUCTION

The exact mechanisms involved in the destruction of the β cell in diabetes are unclear. MSZ treated mice do not develop hyperglycaemia immediately or shortly after treatment, but can take a number of days (Chapter 2). Besides being a β cell toxin, SZ has been found to have antibacterial, antitumour and oncogenic activities (Weiss et al 1982). Furthermore, MSZ treatment in CD-1 mice is followed by the appearance of retrovirus type C within the β-cells (Like and Rossin 1976).

It is now proposed to summarise evidence for the involvement of viruses in the induction of diabetes and also to give a brief outline of the activities of natural killer (NK) cells and antibody dependent cellular cytotoxicity (ADCC) in the destruction of virally infected cells.

Retroviruses are ubiquitous throughout vertebrate species and are maintained in nature as pro-viruses integrated in the host chromosome, thus allowing transmission to the progeny of the host. The presence of type C and type A virus particles has been described in the pancreatic acinar cells of normal mice (Della Torra and Della Porta 1972). Similar detailed studies also revealed that retroviruses could be detected in the β cell of normal CD-1 mice, but that increased expression only occurred after MSZ treatment (Like et al 1977; Appel et al 1978). The mechanisms involved in the activation of the endogenous viral genome to start making, or to increase the

expression of, viral protein are not known, however certain chemicals such as dexamethasone can induce activation (Della Torra and Della Porta 1972; Boiocchi et al 1975).

The role played by the virus in the induction of β-cell damage is not completely understood. The fact that the increased expression of type C virus only occurs in the β cell and not in the other cells of the islet, together with evidence that the appearance of the virus precedes the induction of hyperglycaemia, suggests that there might be a pathological role. Studies attempting to correlate the presence of type C virus in IDDM have not been successful since antibodies to the virus could not be detected in the sera of recently diagnosed diabetics (Muntefering and Jansen 1984). However, other viruses such as encephalomyocarditis (EMC), and Coxsackie B4 have been shown to cause diabetes in humans (Huber and MacPherson 1984), and in mice (Coleman et al 1973; Yoon et al 1978). Coxsackie B4 isolated from the pancreas of a dead diabetic patient was also shown to produce diabetes when inoculated into mice (Yoon et al 1979).

There are several possible mechanisms of beta cell injury in IDDM having a viral aetiology. One mechanism is the direct virus-mediated destruction of infected cells. Another mechanism is when virus replication alone does not cause sufficient damage to cause IDDM but produces enough alteration in the cell membrane to activate immunological attack (Jensen et al 1977).

NK cells and ADCC (K) cells are members of the null or third population of lymphoid

cells, so called because they are of lymphoid origin, but do not carry markers for either T or B cells. They are characterised by the possession of Fc receptors, associated morphologically with the large granular lymphocytes, and display some of the markers of the T cell. They are bone marrow derived cells and exist in normal numbers in animals lacking B and/or T lymphocytes. NK cells non-specifically kill tumour cells or virally infected cells, and play a role in regulating the immune response (Herberman and Ortaldo 1981; Hoffman et al 1986). ADCC cells also kill non-specifically but this occurs via target specific antibody. NK cells have the ability to lyse their target in the absence of prior sensitisation towards structures present on the target cell and are not MHC restricted. Further details of both cell types can be found elsewhere (Wigzell and Ramstedt 1986).

In order to investigate immunological mechanisms involved in the induction of diabetes, it was proposed to investigate NK cell activity and ADCC in MSZ induced diabetes. The rationale for these studies comes from the initial observation by Like and Rossini but also from the fact that viral infection has been shown to cause diabetes (Huber and MacPherson 1984) and also from the fact that NK cells are known to be activated during viral infections (Roitt et al 1985). In the present study, YAC-1 cells, which are virally transformed mouse cells and sensitive to destruction by mouse NK cells (Kiesling et al 1975a and 1975b) were used as target cells. ADCC was investigated by a standard technique employing chicken red blood cells (CRBC) as target cells and anti-CRBC sera as a source for target specific antibody. Effector cells were obtained from the spleen of MSZ treated mice, the spleen having previously been shown to contain a high number of NK cells and high levels of ADCC (Herberman et al 1975a). Cytotoxicity of the target cells was measured by release of ^{51}Cr.

5.2 MATERIALS AND METHODS

Male TO mice were used throughout. Diabetes was induced and assessed as described previously in Chapter 2.

5.2.1 Preparation of effector cells

Splenocytes from age matched control and MSZ treated mice were assayed in parallel 15 days after the first SZ injection. Splenocytes were prepared from individual spleens as described in Chapter 3.

5.2.2. Target cell culture

YAC-1 cells (Flow Laboratories, UK) were grown in suspension using a complete culture medium RPMI-1640. Culture medium preparation and culture condition were as described in Chapter 6. Stock cultures were split every 3 days. YAC-1 cells were stored frozen in liquid nitrogen when not required, as described in Chapter 6. Freshly thawed cells were cultured for at least 3 days before use in the cytotoxicity assay. The culture conditions described produce target cells of high viability (>95%) when assessed by trypan blue dye exclusion.

5.2.3 Labelling YAC-1 cells

YAC-1 cells were labelled with ^{51}Cr according to the method described by Wigzell (1965) with the following modifications. Briefly, $5x10^6$ target cells in 0.5ml of RPMI-1640 were cultured in the presence of 100 µCi $Na_2\,^{51}CrO_4$ (Amersham Ltd., UK) for 60 minutes, with occasional agitation. The cells were then washed 3 times by centrifugation in 10ml of culture medium.

5.2.4 Preparation of CRBC and anti-CRBC antibody

Fresh chicken blood as a source for chicken red blood cells (CRBC) was collected from the wings of adult chickens (Ross Poultries, UK) into heparinised tubes. CRBC were washed 3 times with complete medium and ^{51}Cr labelling as described for YAC-1 cells.

Anti-CRBC sera, as a source for anti-CRBC IgG, was prepared by immunising 10 TO mice with 0.1ml of 10% CRBC (Flow Laboratories, UK) in phosphate buffered saline (v/v). After 14 days the mice were re-immunised with the same dose. Serum from each mouse was collected 10 days following the secondary CRBC challenge and pooled. Standard haemagglutination tests (Chapter 2) revealed that the serum contained a high titre of 2-mercaptoethanol resistant antibodies (IgG) to CRBC.

5.2.5 Measurement of NK cell activity

Cytotoxicity against YAC-1 cells was performed in standard U-shaped microplates (Flow Laboratories, UK) in a total volume of 200 µl. Triplicate cultures of $2x10^4$ target cells were incubated with effector cells to produce effector: target cell (E:T) ratios of 50, 25 and 12.5 : 1. The cells were cultured for 5 hrs at 37°C under 5% CO_2 and 95% humidified air. After incubation, supernatant was collected by supernatant harvester (Flow Laboratories, UK) and the amount of ^{51}Cr release was determined in a γ-counter (Beckman-γ-5500 UK). Specific cytotoxicity was calculated from the mean of triplicate determinations using the following formula:

$$\frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximal cpm} - \text{spontaneous cpm}} \times 100$$

Spontaneous ^{51}Cr release was determined from wells containing ^{51}Cr labelled target cells alone and maximum release from 1% NP40 (Sigma Chemicals Company, UK) lysed target cells. Total label was determined by measuring cpm of $2x10^4$ whole target cells. Spontaneous release was 22% (n=10) of the total label, and maximum release recovered by the supernatant harvester was 70-80% of the total label. Intra-assay variation amongst replicate samples was <5% of the counts.

5.2.6 Antibody-dependent cell-mediated cytotoxicity activity

$2x10^4$ ^{51}Cr-labelled CRBC were incubated with $1x10^6$ spleen cells (E:T ratio 50 : 1) with different dilutions of anti-CRBC IgG in a total culture volume of 200 µl. The cells were cultured in a standard U-shaped microplate for 18hrs at 37°C under 5% Co_2 and 95% humidified air. After incubation, ^{51}Cr released into the supernatant was counted in a γ-counter. Spontaneous release was determined by incubating $2x10^4$ ^{51}Cr labelled target cells with $1x10^6$ spleen cells from MSZ treated mice but in the absence of anti-CRBC antiserum. Maximum release was determined by lysing the labelled target cells with 1% NP40. Spontaneous release was 17% (n=10) of the maximum release. Intra-assay variation amongst replicate samples was <4% of the counts. Specific cytotoxicity was calculated from the mean of triplicate cultures using the formula described in the NK cell assay.

5.3 RESULTS

Figure 5.1 shows the NK-cell activity, as determined by the toxicity of spleen cells towards ^{51}Cr-labelled YAC-1 cells. When measured 15 days after the first SZ injection, NK cell activity was significantly elevated ($\rho < 0.05$) in spleen cells from MSZ treated mice. Raised NK cell activity was found in spleen cells from MSZ treated mice at all the E/T ratios tested. Increasing the E/T ratio was also reflected in an increase in the NK-cell activity.

Figure 5.2 depicts ADCC as determined by the toxicity of spleen cells towards ^{51}Cr-labelled CRBC in the presence of anti-CRBC IgG. As with the NK-cell activity, ADCC was significantly increased in spleen cells from MSZ treated mice. As the IgG titre in the culture was increased ADCC also increased linearly.

Following MSZ treatment, the diabetic status of individual mice was confirmed by glucose estimation. In all cases 15 days after MSZ-treatment the mice were considered hyperglycaemic 27±4 mMol/l, whilst control mice had glucose values of 10±2 mMol/l.

Figure 5.1

Specific lysis of ^{51}Cr labelled YAC-1 cells by lymphocytes from MSZ-treated (shaded bar) and control mice (open bar)
* $P < 0.05$, n=10

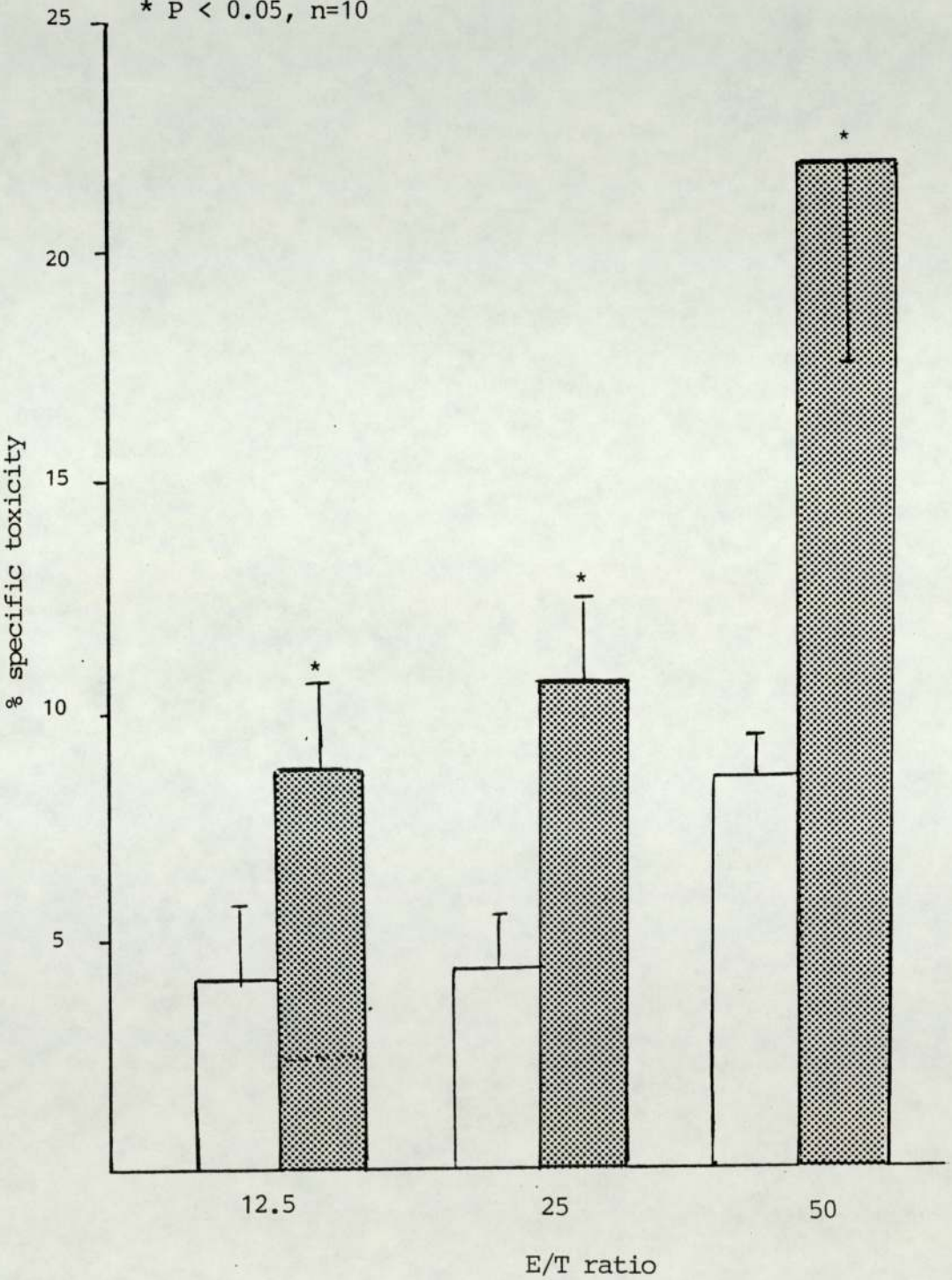
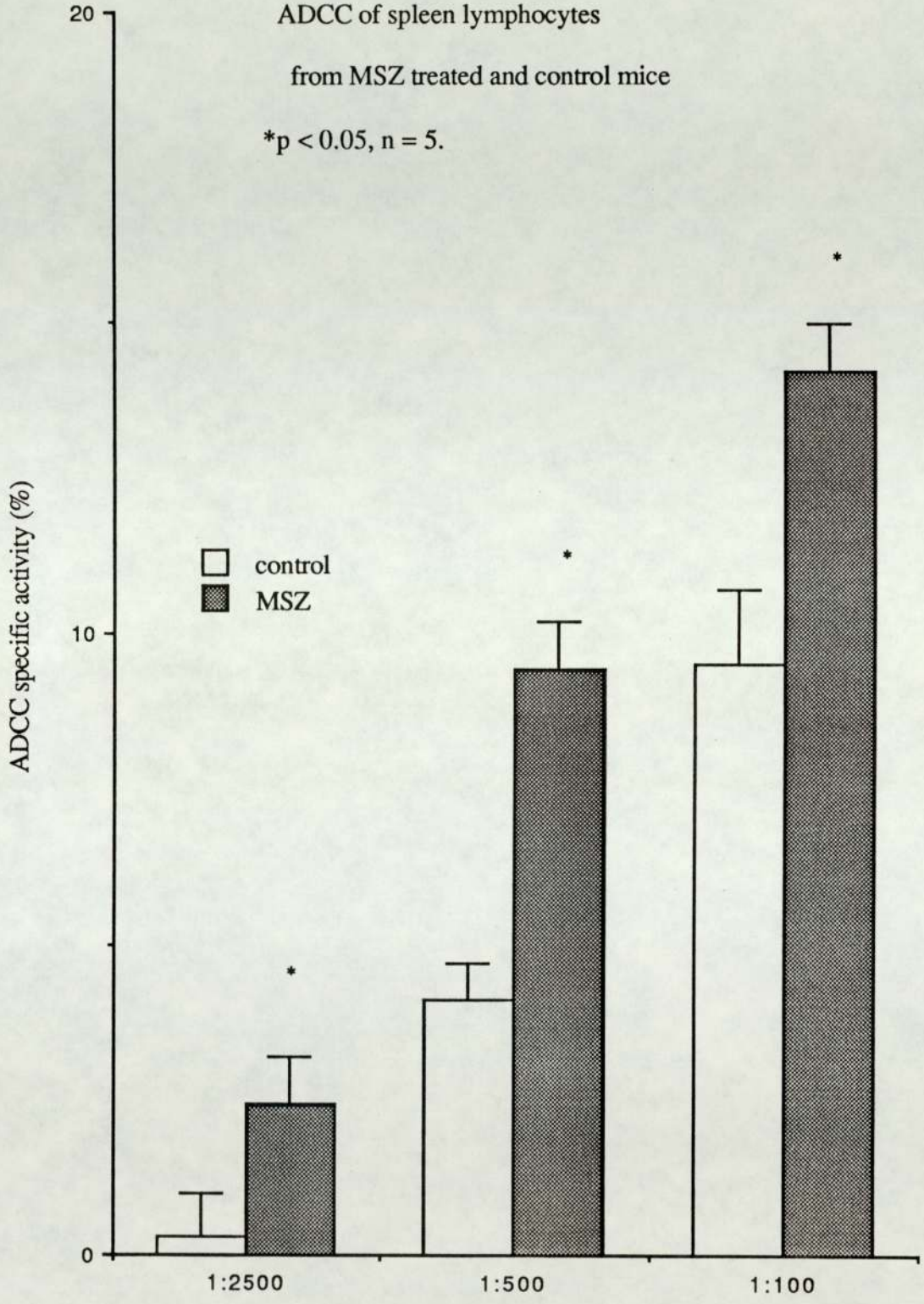


Figure 5.2

ADCC of spleen lymphocytes

from MSZ treated and control mice

*p < 0.05, n = 5.



5.4 DISCUSSION

Work presented in this thesis shows that, when measured 15 days after the first SZ injection, NK cell activity and ADCC were increased in the spleens of MSZ treated TO mice. Whilst there have been no reports on the measurement of ADCC in MSZ treated mice, McEvoy et al (1987) recently reported that MSZ treatments in C57BL/6J mice did not produce elevated NK cell activity. The reasons for these contrasting findings are not apparent, however the discrepancy in the results may be due to differences in methodology including the method of preparation and storage of effector cells. McEvoy et al used NH_4Cl to lyse contaminating RBC from the splenocyte cell preparation, however it has been shown that an increase of intra-cellular pH in NK cells (which would be observed when NH_4Cl is used) depresses the lytic activity of these cells (Eremin and Plumb, 1978). During preparation of effector cells it is also important to store the cells at 0°C to retain lytic activity (Herberman et al 1975b), however, it is not clear whether McEvoy et al did this. McEvoy et al measured NK cell activity 10 days after the first SZ injection, whilst our data were obtained after 15 days. In a preliminary study we found that NK cell activity was normal at 10 days post MSZ treatment. It may be therefore that McEvoy et al looked too early for changes in NK cell activity.

The work present in this investigation is of interest in the light of recent reports of the presence of NK cells amongst the inflammatory cells in the islets of MSZ treated mice (Schneider et al 1984; Cossel et al 1985). These results implicate a pathological role for NK cells in causing diabetes. It could be that type C virus induced by MSZ

treatment in the β cell, activates NK cells. Following activation, in an attempt to protect the host against the virus, NK cells inadvertently kill β cells and cause diabetes.

It has been found that as well as K cells, NK cells are also capable of ADCC. Moreover, some authors suggest that NK cell activity and ADCC may be performed by only one cell type (Kimber 1985). In the light of the above, the fact that ADCC was found to be increased in MSZ treated mice may be a consequence of elevated NK cell activity. ADCC has been reported to be elevated in Type I diabetics (Pozzilli et al 1979; Sensi et al 1981; Richens et al 1982). Pozzilli et al reported that 57% of newly diagnosed diabetics had elevated ADCC but this decreased to 16% when measured between 6 months and 2 years after diagnosis. The significance of ADCC in-vivo is unclear. Whilst the present study used CRBC as the target cell, there have been recent reports which found raised ADCC in IDDM when using islet cells as the targets together with islet cell antibody (Kohler et al 1984; Charles et al 1984). These results suggest a pathological role for ADCC in the induction of diabetes.

NK cell activity and NK cell number has been reported to be decreased in recently diagnosed Type I diabetics (Negishi et al 1976). Since a viral aetiology for Type I diabetes has been postulated (Huber and MacPherson 1984), and NK cell activity has been shown to be increased following virus infection (Roitt et al 1985), it might follow that NK-cell activity should be elevated in Type I diabetes, but the above studies have not supported this proposal. The lack of NK cell activity in IDDM could imply that either elevated NK cell activity is not an important factor in the pathogenesis

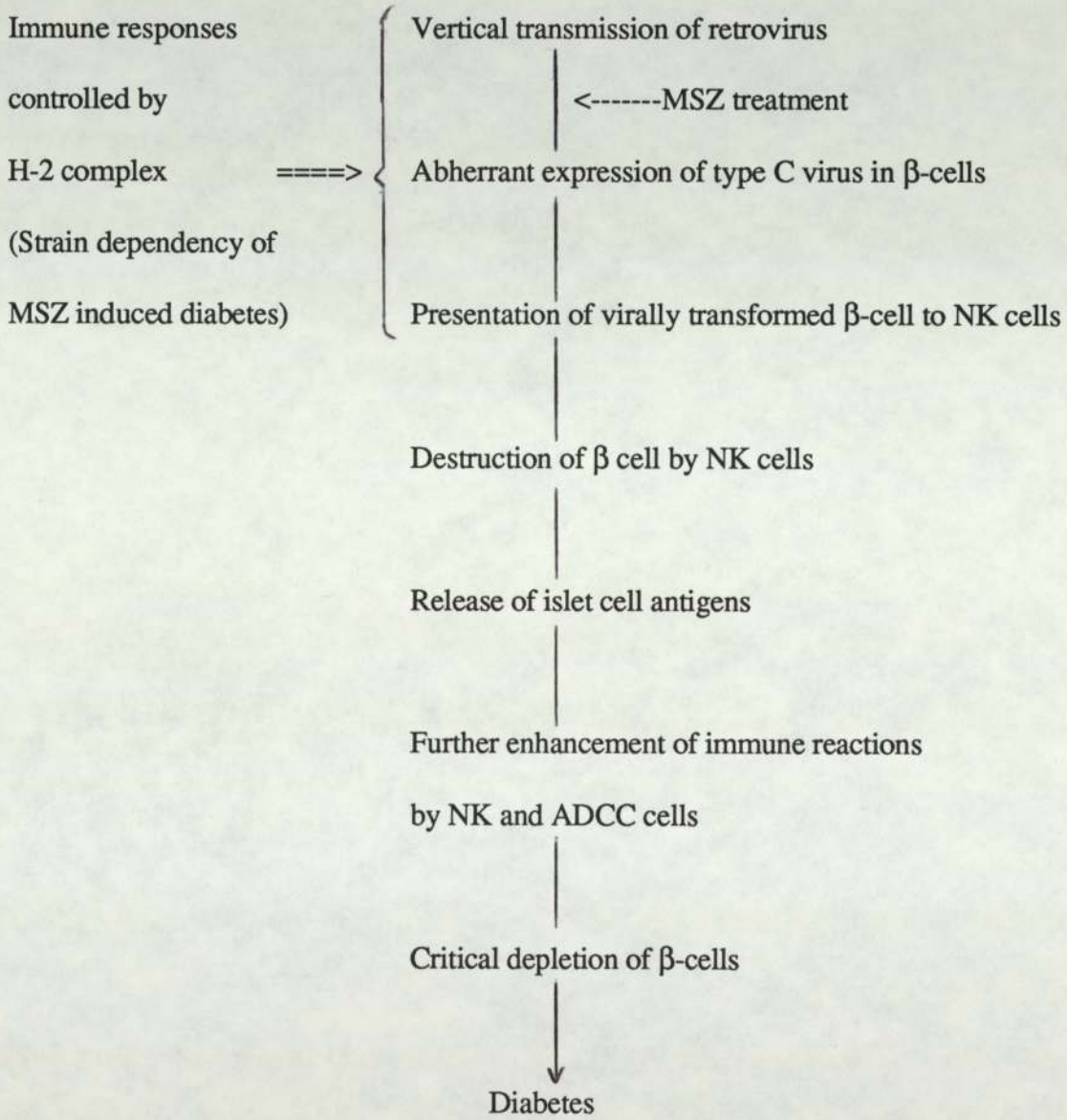
of diabetes or that the studies did not pick up enhanced NK cell activity because the investigators did not examine very recently diagnosed Type I diabetics. Evidence for the latter proposal comes from the report which found increased NK cell activity in the early phases of a viral infection but decreased NK cell activity during the latter stages of the infection (Roitt et al 1985). It could be that those investigators who have reported decreased NK cell activities in diabetes looked too late. It would be interesting therefore to examine NK cell activities before the onset of the clinical disease during the period of proposed viral expression and increased NK cell activity.

With regard to the strain susceptibility (Chapter 2) and male dependence (Rossini et al 1978a; Wolf et al 1984) of MSZ induced diabetes in mice, it is interesting to note that NK cell activities in mice are also dependent on genetic strain (Kiessling et al 1975). Similarly, in humans, the MHC has been shown to influence the level of NK cell activity, thus low NK cell activity was found in HLA-B7 positive individuals (Santoli et al 1976). Also the frequency of HLA-B7 amongst IDDM patients was lower than expected (Cudworth and Wolf 1984). The incidence of IDDM in male children is more frequent than females (DeBeaufort et al 1988). Furthermore, NK cell activity is higher in males than in females (Pross and Bains, 1982). Clearly more work is required to establish whether the relationships between NK cell activity, HLA and gender are circumstantial, or whether any of these relationships are also important in the induction of diabetes.

To summarise, the present study found that NK and ADCC was increased in MSZ

treated mice. These cells may play a significant role in inducing diabetes, a proposed mechanism for their involvement is described in Figure 5.3. However, further studies are required to fully establish this. The work presented here should firstly be extended to determine how peak NK and ADCC corresponds to the time of onset of diabetes. The nature of these in-vitro assays should also be related to the in-vivo situation, and the nature of the infiltrating cells in insulitis needs to be more clearly defined, this could be achieved by suitable monoclonal antibody markers. Certain agents, such as interferon, are known to augment NK cell activity (Santoli and Koprowski 1979). Such treatment might also prove to augment MSZ induced diabetes in mice. Similarly, it would be interesting to investigate whether mice having impaired NK cell activities, (such as the beige mouse Roder and Duwe, 1979), are resistant to MSZ induced diabetes.

Figure 5.3 Proposed mechanism for the involvement of NK cell activity and ADCC in MSZ treated mice.



CHAPTER 6: CELL AND HUMORAL IMMUNITY AGAINST THE β CELL

6.1 INTRODUCTION

Several lines of evidence have suggested that autoimmune reactions might be involved in the pathogenesis of IDDM in humans as well as certain animal models (for review see Drell and Notkins 1987).

IDDM is characterised by the destruction of β cells. Although the exact mechanisms that lead to this destruction are not clear, immune factors have been suggested on the basis of the following findings: (1) the presence of inflammatory cells in the islets; (2) the detection of ICA, ICSA and other autoantibodies (e.g. anti-DNA and anti-thyroid) in the sera of diabetic patients; (3) the finding that cell-mediated immune mechanisms may contribute to the destruction of β cells and (4) immune intervention studies (see Chapter 1).

Diabetes induced in response to MSZ treatment is characterised by insulitis and delayed onset of hyperglycaemia in susceptible strains of mice (Like and Rossini, 1977). Since insulitis is also a histopathological finding associated with IDDM and indicative of a possible autoimmune reaction (see Chapter 1) it has been suggested that MSZ-induced diabetes might also have an autoimmune aetiology. Consequently, it was important to establish whether insulitis was directly associated with hyperglycaemia and if so, to determine if functional T-lymphocytes involved in insulitis were necessary for the induction of diabetes. Studies which attempted to

resolve this issue have produced conflicting results. An immune component in the pathogenesis of MSZ induced diabetes was supported when it was shown that injection of rabbit antiserum against mouse T-lymphocytes (ALS) prevented MSZ induced diabetes (Rossini et al 1978b). Further support for the involvement of cell mediated immunity came when Buschard and Rygard (1978a) showed that athymic nude mice were resistant to MSZ induced diabetes, whilst genetically similar mice with an intact thymus and cellular immune responses were susceptible. Subsequently Paik et al (1980) confirmed these observations and suggested that T-cell functions were obligatory for disease susceptibility since nude mice became susceptible to MSZ induced diabetes when T-cell function was reconstituted by thymus grafts. Furthermore, Paik et al (1982) also reported that irradiation (which destroys functioning lymphocytes) prevented MSZ induced diabetes, and reconstitution of irradiated mice with T-lymphocytes, but not B-lymphocytes, restored the susceptibility to MSZ treatment. In contrast however, there have been reports which found that nude mice were sensitive to MSZ induced diabetes (Beattie et al 1980; Leiter 1982). Leiter et al (1982 and 1983) also reported than an MSZ susceptible strain of mice remained susceptible following neonatal thymectomy or adolescent thymectomy and irradiation.

Further evidence of the role of CMI in the induction of diabetes in MSZ treated mice comes from passive transfer studies. It was reported that splenic lymphocytes from MSZ treated diabetic mice could transfer diabetes to either syngeneic normal or nude mice (Buschard and Rygaard (1977 and 1978). However, these studies have not been reproducible (Beattie et al 1980).

The exact role played by insulitis in causing hyperglycaemia is therefore not clear. There are reports which suggest that it is a prerequisite for hyperglycaemia (Nakamura et al 1984), however, there have been observation which describe insulitis in the absence of hyperglycaemia (Sandler 1984). Bonnevie-Nielsen et al (1981) reported that after MSZ treatment the majority of β cell death occurred before insulitis was apparent. Leiter et al (1983) concluded that insulitis was a consequence of β cell death rather than its cause.

Clearly, there is considerable disagreement on whether an autoimmune component is involved in the induction of hyperglycaemia by MSZ treatment. There are, of course, strain differences in susceptibility to MSZ treatment, and this might explain some of the conflicting data. On the other hand, it might be that hyperglycaemia is produced as a direct consequence of SZ toxicity on the β cell (Nichols et al 1979; Bonnvie-Nielsen et al 1981; Sandler et al 1984) and autoimmune reactions are not required.

The purpose of the present study therefore, was to investigate, firstly, if insulitis associated with hyperglycaemia was present in the TO mice model used here, and secondly, to determine if anti-pancreatic active lymphocytes are induced by MSZ treatment. A number of techniques were employed using lymphocytes isolated from MSZ treated mice. These included an assessment of cytotoxicity of lymphocytes from MSZ treated mice, against ^{51}Cr-labelled islet cells; a study of the effect of lymphocytes on insulin release from an insulin-secreting cell line, HIT-T15; and the measurement of proliferation in lymphocytes isolated from MSZ treated mice in the presence of either an homogenate of HIT-T15 cells or mitomycin-C-treated, but intact, HIT-T15 cells. Finally, anti β cell humoral responses were examined by determining whether ICSA could be detected in the sera of MSZ treated mice.

6.2 MATERIALS AND METHODS

10 week old male TO mice were used throughout. Diabetes was induced as described in Chapter 2 and assessed by glucose determination in each experimental group.

6.2.1 Materials

Analytical grade reagents and double distilled water were used throughout, suppliers of reagents were as described in Chapter 2.

Additional material was obtained as described below:

HIT-T15 cells were kindly provided by Dr.R.F.Santerre of Eli Lilly Ltd., USA.

RINm5F cells were kindly donated by Dr.S.J.H.Ashcroft from The John John Radcliffe Infirmary, Oxford.

Worthington collagenase Class IV (145 μ/mg) was purchased from Cooper Biomedical Ltd., UK.

Anti-mouse IgG Fluorescein Isocyanate (FITC) conjugate was purchased from Sigma Chemical Company, UK.

6.2.2 Detection of insulitis

Pancreata from 5 MSZ treated and 5 control mice were removed on the 17th day following the first SZ injection, this time period was chosen since a previous report had indicated that maximal mononuclear cell infiltration occurs at this stage (Cossel et al 1985). Individual pancreata were fixed in Bouin's solution and embedded in wax. 5 μm serial sections were cut and stained with haematoxylin and eosin (H & E). 5 slides

(which had 3 sections per slide) were selected from the head through to the tail of the pancreata, insulitis was scored when mononuclear lymphocytes were observed in and around islets after examination with a light microscope.

6.2.3 Immunological assays

All immunological assays employed in the present study were performed on mice 10 days after the first SZ injection. Parallel assays were set up for control, vehicle injected mice. Splenocytes were prepared as described in Chapter 3. In the ^{51}Cr-release and insulin release assay splenocytes were further purified by centrifugation through Ficoll-Hypaque (Boyum 1968). Serum was prepared from blood collected from the heart immediately following cervical dislocation of the mice.

Conventional immunological techniques (^{51}Cr release, insulin release and indirect immunofluorescence) have been valuable in the discovery and initial characterisation of cytotoxic lymphocytes and ICSA (Dobersen et al 1980; Boitard et al 1981; Charles et al 1983; Itoh et al 1984). However, islets or islet cell preparations are a heterogeneous cell population consisting not only of insulin secreting β-cells, but other endocrine cells, as well as fibroblasts and pancreatic ductal cells. Insulin released from the β-cells in response to various trophic stimuli therefore, would be the net result of the paracrine effects of glucagon, somatostatin and pancreatic polypeptide released from adjacent cells within the islet. As a consequence of this, some restrictions have to be imposed on the interpretation of in-vitro studies involving insulin release. A fibrous capsule surrounds the islet, and this physical barrier could be disadvantageous in studies involved with the detection of cytotoxic lymphocytes

where a cell to cell contact is required. A pure β-cell preparation would resolve these issues, enriched preparations of β-cells can be achieved e.g. by the use of fluorescent activated cell sorter (reviewed by Pipeleer et al 1987) however, this is often unavailable to most researcheres because of the high costs involved.

In the present study hamster derived SV40 virus transformed β cell line (HIT-T15) were used as an alternative to isolated islets. This cell line has been characterised in our laboratories. Although HIT-T15 cells are unphysiological - the maximal insulin release of HIT-T15 cells has been reported to be in the range of 7.5-10 mMol/l glucose (Santerre et al 1981; Ashcroft et al 1986; see also studies reported from our laboratory in Lambert et al 1986). The stimulation of HIT-T15 cells compares only modestly when maximal stimulation from isolated mouse islets occurs at higher glucose concentrations in the range of 16.7-22.2 mMol/l(Ashcroft et al (1972). Nevertheless, HIT-T15 cells have advantages over isolated islets because they are dissociated from the islet paracrine micro-environment and no barriers are present. Also HIT-T15 cells can be grown in tissue culture, this can provide essentially unlimited amounts of tissue. They are pure β-cells (virally transformed) and release only insulin, Lambert et al (1986)was unable to detect other hormones such as glucagon. In this regard they have advantages over the rat insulinoma continuous cell line because these, release insulin as well as other hormones and are relatively unresponsive to physiological levels of glucose (Gazdar et al 1980).

6.2.4 Isolation of obese mouse islets of Langerhans using collagenase digestion

Pancreatic islets were isolated from obese (ob/ob) mice by a modification of the technique first described by Lacy and Kostianovsky (1967). Mice were killed by

cervical dislocation, positioned ventral side up and the viscera displayed using a mid ventral incision. The viscera were displaced to the left to reveal the full extent of the pancreas. The latter was distended with Krebs/HEPES buffer (pH 7.4) using a syringe and a 24 gauge needle and removed from its point of contact with the stomach and duodenal loop with butterfly scissors. The excised pancreas was trimmed of fat and connective tissue, washed and cut into small pieces with round ended scissors. Pieces of pancreas derived from two mice were transferred to a conical flask (25ml) containing 8ml of Krebs/HEPES buffer (Appendix A2), and 40mg of Worthington collagenase. The contents were then vigorously shaken in a shaking water bath (120 cycles/minute) at 37°C for 20-30 minutes. The digest was subsequently made up to 15ml with ice cold Krebs/HEPES buffer to prevent any further digestion. The contents were then transferred to a centrifuge tube (15ml) and allowed to sediment. This procedure was repeated to remove all traces of collagenase. The final sediment was re-suspended in 10ml of Krebs/HEPES buffer and transferred to a glass petri dish. At this stage DNA-ase (200µg/digest) was added to prevent clumping of the debris. Islets free from acinar tissue could be picked out using a pasteur pipette under a dissecting microscope (magnification X10).

Islets were cultured overnight in supplemented RPMI-1640 culture medium. Following culture, batches of 50-100 islets were disaggregated for 3 minutes with trypsin/EDTA to yield a single cell suspension. The single cell suspension was then ready for ^{51}Cr release cytotoxicity assay. Viability of single cell suspensions, as determined by trypan blue exclusion test, was >75%.

6.2.5 Maintenance of cell cultures

HIT-T15 and RINm5F cells were maintained in (RPMI-1640) tissue culture medium supplemented with 10% heat inactivated (56°C for 30 minutes) foetal calf serum, glutamine (1 mMol/l), penicillin (100 IU/ml), streptomycin (100µg/ml) and HEPES (10m Mol/l). Cultured cells were grown in costar tissue culture flasks (250ml).

Cells were routinely passaged each week and were fed twice weekly with fresh culture medium. All cultures were maintained at 37°C in 5% CO_2 / humidified air, using a temperature controlled CO_2 incubator (Flow Laboratories, UK). All experimental work was performed between passages 47-70. Flasks to be passaged were rocked gently to free any loose or dead cells. The spent medium was removed and the flasks containing adherent cells were rinsed with 9ml of trypsin/EDTA (0.05% w/v, 0.02% w/v). 1ml of trypsin/EDTA was then added and the flasks were incubated for 6-10 minutes to dislodge the cells. After the incubation, serum supplemented culture medium was added to the flasks and the resulting cell suspensions were centrifuged at 150g (MSE Chillspin) for 3 minutes. The cell pellets were re-suspended in 5ml of culture medium and cells were enumerated using a haemocytometer. Re-seeding was carried out at $4x10^6$ cells per 250ml Costar tissue culture flask containing 20ml of culture medium.

6.2.6 Media preparation

RPMI-1640 and HBSS were prepared from powdered media. The contents of one bottle (sufficient to make up 1L) were dissolved in double distilled water, 2.0g/l sodium bicarbonate and 10 mMol/l HEPES were added. The pH was adjusted with 1 Mol/l HCl to pH 7.4. Sterilization was achieved by membrane filtration using a pump

and 0.22μm filter (Millipore, UK). Batch sterility was monitored by incubation of the medium at 37°C in a vented petri dish, any batches showing bacterial contamination were discarded.

6.2.7 Cryopreservation of cells

The preservation of cell cultures in the frozen state offers several advantages (Scherer 1965). This procedure enables multiple experiments to be performed upon the same cell stock and permits storage of stock cells when they are not routinely required. The principles underlying cryopreservation can be found elsewhere (Taylor and Benton 1987). In the present study HIT-T15, RINm5F and YAC-1 cells (Chapter 5) were frozen in liquid nitrogen by the following procedure:

4-6 x 10^6 cells from freshly trypsinised stocks were suspended in 1ml of supplemented tissue culture medium containing 10% DMSO and placed in 1.2ml ampoules (Nunc Gibco Ltd., UK). Ampoules were pre-chilled to 4°C in a refrigerator for 20-30 minutes. The chilled ampoules were loaded into a Handi Freeze 35HC freezing tray (Union Carbide, Indianapolis, USA), which was then placed into the neck tube of a Union Carbide 35HC liquid nitrogen freezer. After 20 minutes, ampoules frozen down to -65°C were loaded into canes and plunged into liquid nitrogen at -196°C for storage.

When required ampoules were rapidly thawed in a water bath at 37°C. The contents of the ampoules were transferred into 30ml of culture medium. Medium was replaced after 24 hours and cell culture was continued as described. Post-freezing viability was

>70% when assessed by trypan blue dye exclusion.

6.2.8 Cellular cytoxicity by ^{51}Cr-release

Obese mice islet cells were labelled with ^{51}Cr as described previously for YAC-1 cells in Chapter 5. ^{51}Cr-labelled islet cells (2 x 10^4) were then co-cultured with splenocytes in 0.2ml of supplemented RPMI-1640 at effector : target ratios of 50, 25 and 12.5:1, for 5 hours at 37°C in 5% CO_2/humidified air. Collection of supernatant, measurement of ^{51}Cr-release and expression of results were as described for the NK-cell assay (Chapter 5).

6.2.9 Effect of lymphocytes on HIT-T15 cell insulin release, a measurement of cellular cytotoxicity

This experiment was designed to determine the possible cytotoxicity of splenocytes from MSZ-treated mice on HIT-T15 cells. The method described below is a modification of Lohmann et al 1986).

2 x 10^4 HIT-T15 cells in 0.2ml supplemented RPMI-1640 were seeded into each well of a 96-well tissue culture plate (Flow Laboratories, UK). The cells were allowed to form a monolayer by culturing overnight at 37°C in 5% CO_2/air. This regimen was used to allow normal contact between the cells in the monolayer, which is believed to be important in insulin release (Halban et al 1982). Monolayers were washed in Krebs/HEPES buffer (pH 7.4) supplemented with 0.2g/l BSA and 5.6 mMol/l glucose (Hussain and Atkins, 1985). This concentration of glucose stimulates HIT-T15 cells to release insulin; but at a sub-optimal level (Lambert et al 1986) but was necessarily added to maintain the splenocytes. Bicarbonate was included to prevent

alkalisation and impairment of insulin release (Lindstrom and Sehlin, 1986). After washing, splenocytes (in 0.2ml Krebs/HEPES, supplemented as described above), were added to the wells to produce effector : target cell ratios of 12.5, 50 and 100:1, and incubated for 4 hours. At the end of the co-culture the lymphocytes were washed away with Krebs/HEPES. The remaining attached monolayers were used to assess the capacity for glucose stimulated insulin release using Krebs/HEPES supplemented with 7.5 mMol/l glucose over a 90 minute incubation period. This level of glucose has previously been reported to maximally stimulate HIT-T15 cells. After incubation, a small aliquot was removed and centrifuged to remove any detached cells and then stored at -20°C for insulin radioimmunoassay (Chapter 2). Insulin release was determined from wells previously incubated without lymphocytes but with/without the presence of glucose. Maximum insulin release was determined by lysing HIT-T15 cells with 1% NP40. Incubations were carried out in triplicate and the insulin assay was performed in duplicate, using a human insulin standard. Results are expressed as μU insulin/ml released in the medium.

6.2.10 Preparation of homogenate and mitomycin C treated HIT-T15 cells

5-10 x 10^7 HIT-T15 cells in HBSS were disrupted by sonication on ice, with five 10 sec, 4 A bursts using a sonicator (MSE Soniprep 150 UK). Protein content was determined by the Biuret method.

HIT-T15 cells were treated with mitomycin C following the method described by Swain (1980). In a preliminary study it was found that mitomycin C treated cells did not proliferate when assessed by ^{3}H-thymidine incorporation.

6.2.11 Measurement of lymphocyte proliferation in response to HIT-T15 cells

The response of lymphocytes against an homogenate prepared from HIT-T15 cells, described below, was measured by a modification of the method used by Itoh et al (1984). These authors however, used an homogenate of whole rat islet. The proliferation of lymphocytes against whole mitomycin C treated HIT-T15 (i.e. mixed cell response) was a modification of the mixed lymphocyte response (MLR), a commonly employed in-vitro assay (Bradley, 1980).

5×10^5 spleen cells in supplemented RPMI-1640 were cultured with either a homogenate prepared from sonicated HIT-T15 cells at final protein concentration of 0.5, 0.1 and 0.05 μg protein/ml, or 2×10^4 whole mitomycin C treated HIT-T15 cells. After 52 hours, the cultures were pulsed with 1.0 μCi/well ^{3}H-thymidine and harvested 24 hours later. Harvesting, counting and expression of results was as described previously in Chapter 4.

6.2.12 Detection of ICSA

In the present study RINm5F cells were used as target cells in a modified indirect immunofluorescence method first described by Lernmark et al (1978). Briefly, 5×10^5 RINmSF cells were suspended in HBSS supplemented with 10% heat inactivated FCS (HBSS-FCS), and washed by centrifugation at 300g for 5 minutes. The cells were then incubated with 50μl of test serum diluted 1:2 with HBSS-FCS for 45 minutes at 4°C. The test serum was heat inactivated (56°C for 30 minutes) to prevent the possibility of complement mediated antibody dependent lysis (Rittenhouse et al 1980; Cavender et al 1986). The cells were then ultracentrifuged at 100,000 x g for 12 minutes in a Beckman Airfuge, to minimise non-specific staining (Mishell and

Shiigi 1980). After washing twice with HBSS-FCS, as described above, the cells were subsequently incubated with 50µl FITC-conjugated goat anti-mouse IgG diluted 1:5 with HBSS-FCS for 30 minutes at 37°C. The FITC-antibody conjugate was previously titrated and the concentration giving minimal non-specific binding but effective staining, was used. After the incubation the cells were washed twice with HBSS-FCS, resuspended in FCS and smeared onto a glass slide. Slides were dried, fixed in 1% paraformaldehyde for 30 minutes and then mounted in glycerol-phosphate buffered saline (9:1 v/v). Fluorescent cells were examined by a light microscope fitted with a UV light source (Carl Zeiss Scientific Instruments Ltd., UK). Sera was assessed positive for ICSA when at least 10% of 50-100 cells examined showed fluorescence (Itoh et al 1984). In an initial attempt to quantitate the levels of ICSA present in the sera the number of fluorescent cells following incubation with sera from control and MSZ-treated mice were determined.

6.3 RESULTS

Glucose levels in individual mice were always estimated before each study. When glucose levels were estimated on day 10, typically, there was no significant difference between MSZ-treated mice 11±2 mMol/l and control mice 10±1 mMol/l. Glucose levels however were significantly higher in MSZ-treated mice (30±3 mMol/l) when measured on day 17 when compared to those values in control mice 9±2 mMol/l.

6.3.1 Examination of pancreas

Histological examination of the pancreata following MSZ treatment showed a striking difference in the appearance of the islets between the control and MSZ treated mice. Four out of five mice that had received MSZ treatment showed signs of insulitis. Insulitis was scored when islets observed in several tissue sections showed infiltrating leucocytic cells (Figure 6.1). Heavy mononuclear cell infiltration was found in and around islets, but not in the acinar tissue. Some of the islets in MSZ treated mice were clearly disrupted, however, there were also some islets that were visually indistinguishable from normal islets.

6.3.2 ^{51}Cr release assay

Figure 6.2 shows that the % specific lysis of obese islet cells by splenocytes from MSZ treated mice was significantly ($\rho < 0.05$) increased above the control values. This trend was apparent throughout the ET ratios tested, and peak cytotoxicity was observed at an ET ratio of 25. Some of the cytotoxicity values recorded from control splenocytes gave negative values, this may be due to the uptake of spontaneously released ^{51}Cr from target cells by the splenocytes during the culture (Mishell and

Shiigi, 1980). Spontaneous release of ^{51}Cr by target cells cultured alone was 38% of maximal release. Intra-assay variation was <5% of cpm values.

6.3.3 Insulin release assay

Figure 6.3 shows the insulin release of HIT-T15 cells in response to glucose following exposure with lymphocytes, from either control or MSZ treated mice. Insulin release was significantly lower in cultures which had previously been exposed to splenocytes from MSZ-treated mice. This trend was seen throughout the ET ratios tested, the lowest amount of insulin detected was seen with splenocytes from MSZ-treated mice with the highest ET ratio, this suggested that splenocytes were mediating cell killing. Insulin release by HIT-T15 cells in the presence of lymphocytes from control mice was similar (36.5± 2.5uU insulin/ml) to that seen in HIT-T15 cells cultured alone, with 7.5 mMol glucose, however the basal insulin release from HIT-T15 cells cultured alone without glucose was 23.3±2.8 μU insulin/ml . Maximal release by NP40 lysed HIT-T15 cells was 723 ± 118 μU insulin/ml. These results suggested that HIT-T15 cells were not exhausted and still modestly responded to glucose.

6.3.4 Lymphocyte proliferation assay

Figures 6.4 and 6.5 depict the proliferation of lymphocytes isolated from either control or MSZ treated mice against a homogenate of HIT-T15 cells or whole mitomycin C treated HIT-T15 cells. The stimulation index of lymphocytes from MSZ treated mice was significantly higher at a protein concentration of 0.1 and 0.5μg protein/ml when compared to the control lymphocyte values. However, at a lower

protein concentration, 0.05μg protein/ml, the stimulation index was not significantly elevated (Figure 6.4). Generally stimulation indices produced by lymphocytes co-cultured with whole HIT-T15 cells were higher than the stimulation indices produced by HIT-T15 cell homogenates.

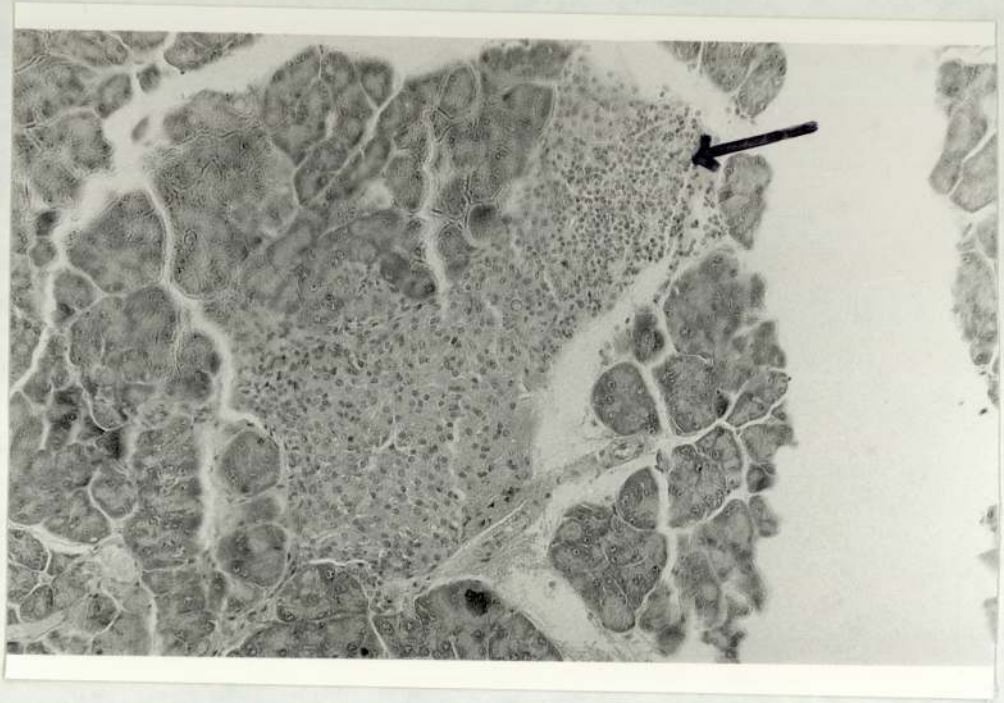
6.3.5 ICSA

Figure 6.6 shows the presence of ICSA in the sera of MSZ treated mice. When serum from 10 MSZ treated mice was examined, 8 mice were positive for ICSA. Serum from 10 control mice was negative for ICSA.Sera was assessed posotive for ICSA when at least 10% of 50-100 cells examined showed Fluorescence.In this semiquantitative assay the exact numbers of fluorescent cells was quantitated.

In an initial attempt to quantitate the measurement of ICSA, target cells demonstrating fluorescence following incubation with sera from control was 4±2% (n=4), whereas the 'mean' value from treated mice was 22% (n=2).

Figure 6.1 Insulitis in MSZ treated mice (A). Note the presence of infiltrating mononuclear cells (arrow) in the islet and their absence from the exocrine tissue .A photograph of an islet from a control mouse is also shown (B) for comparison . (H & E, X 250)

A



B

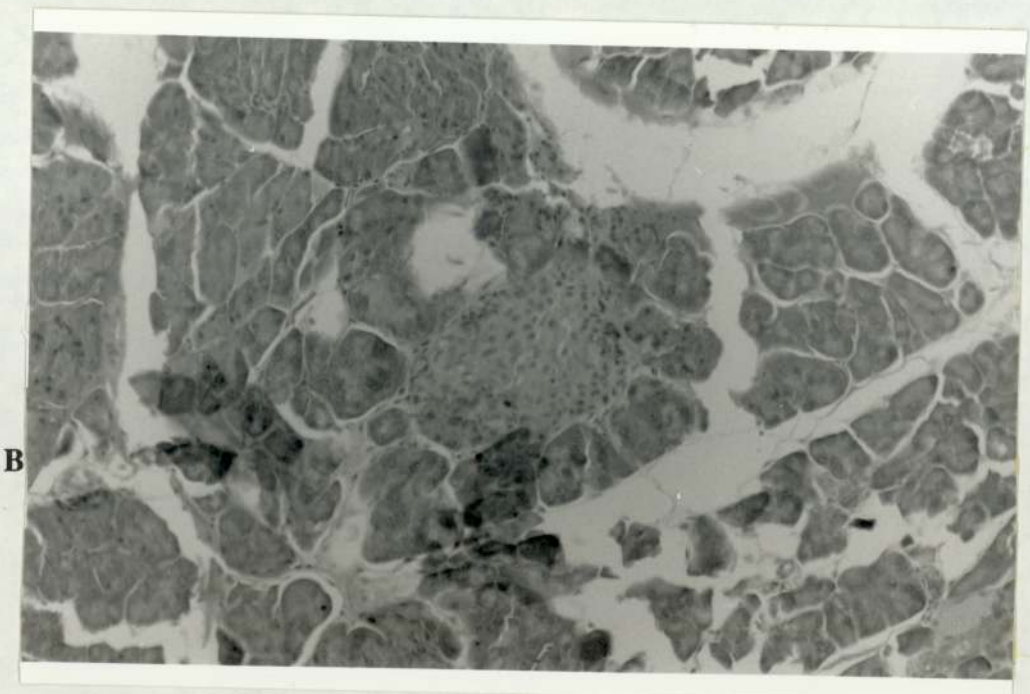


Figure 6.2

Specific lysis of ^{51}Cr labelled obese islet cells by lymphocytes from MSZ-treated mice (shaded bar) and control mice (open bars)
* $P < 0.05$, n=5

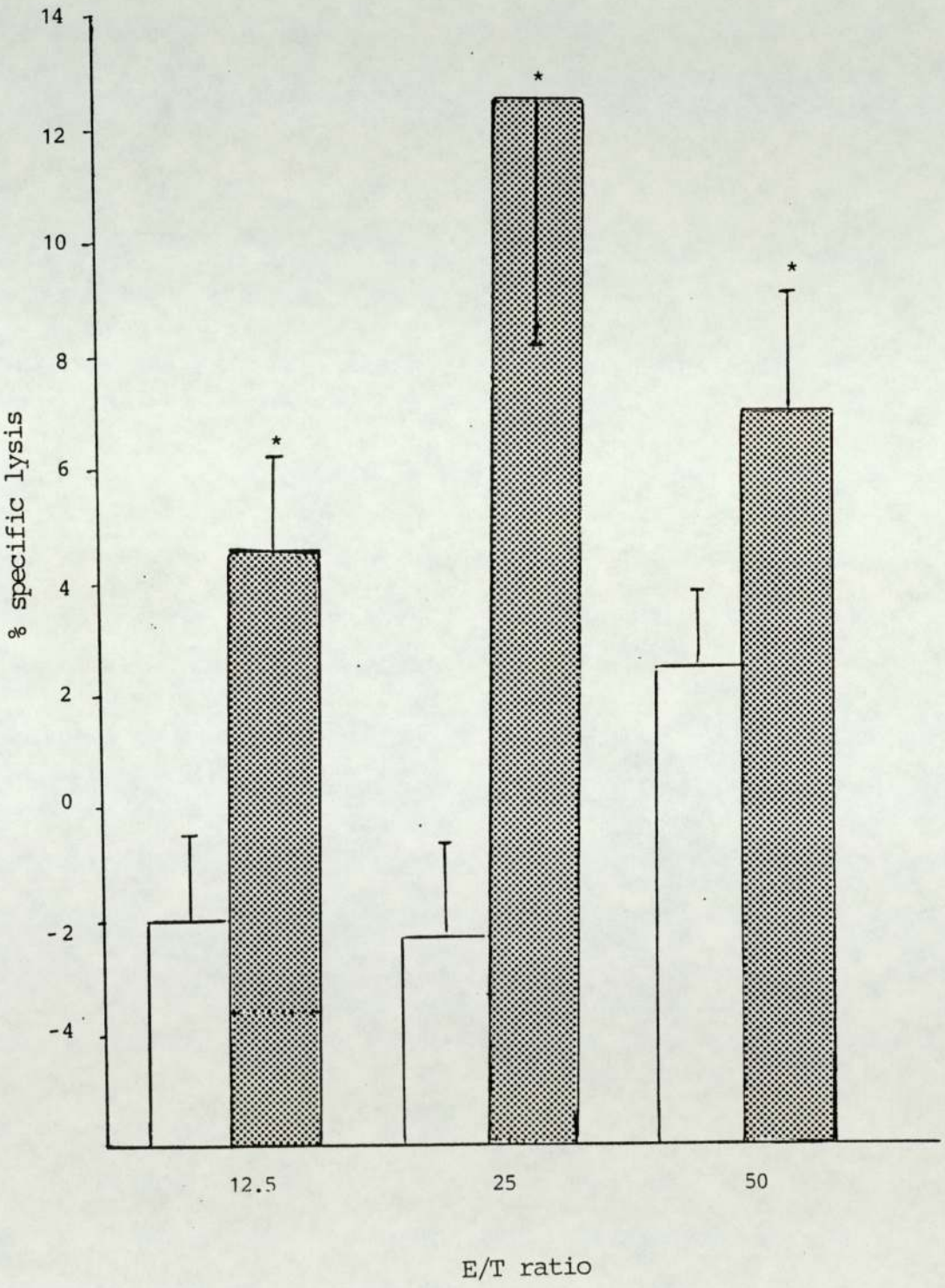


Figure 6.3

Insulin release from HIT-TI5 cells after culture with lymphocytes from MSZ-treated mice (shaded bar) and control mice (open bar) * $P < 0.05$, n=9

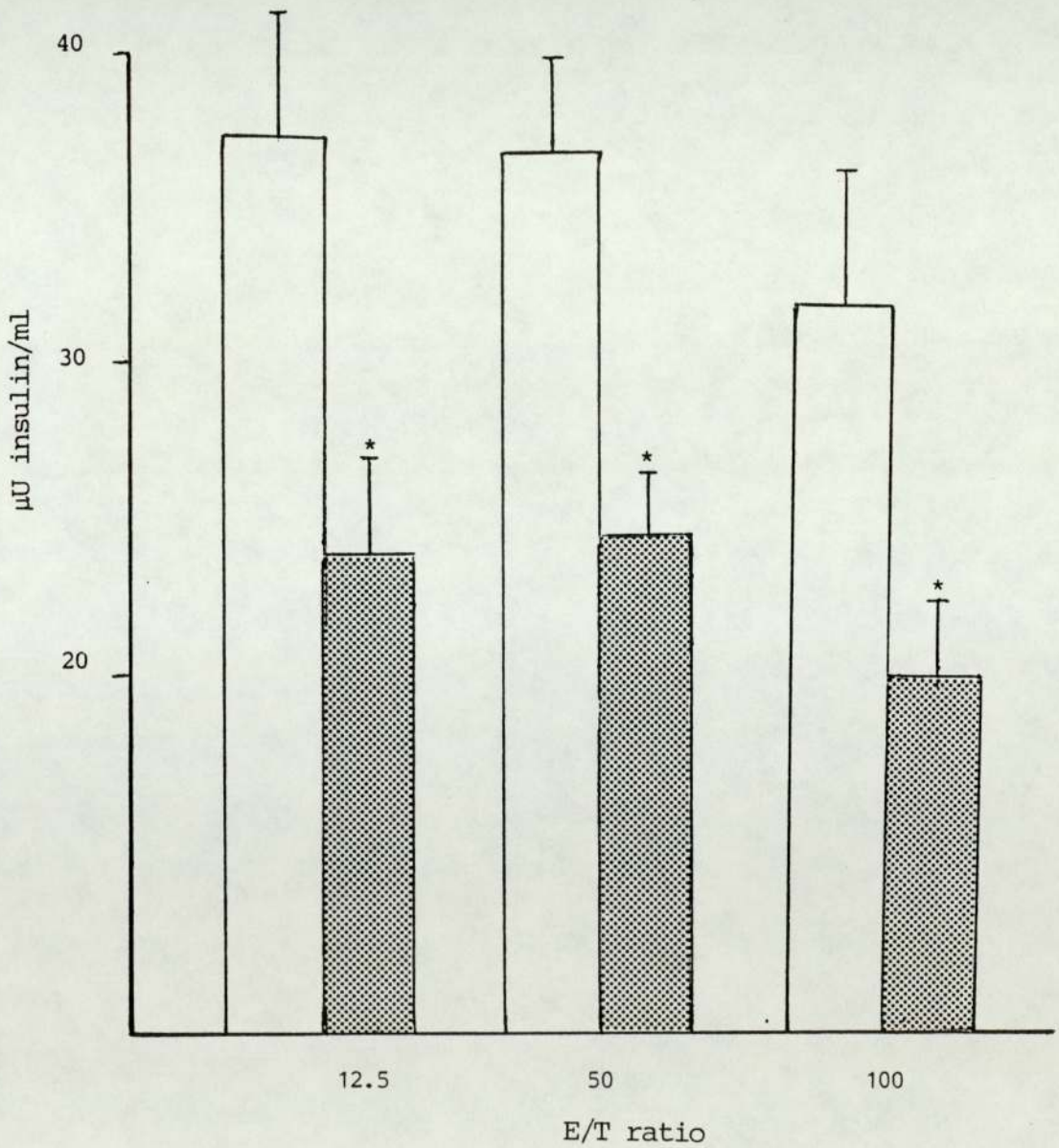


Figure 6.4

Proliferation of lymphocytes from MSZ-treated mice (shaded bar) and control mice (open bar) by an homogenate of HIT-TI5 cells
* $P < 0.05$, n=5

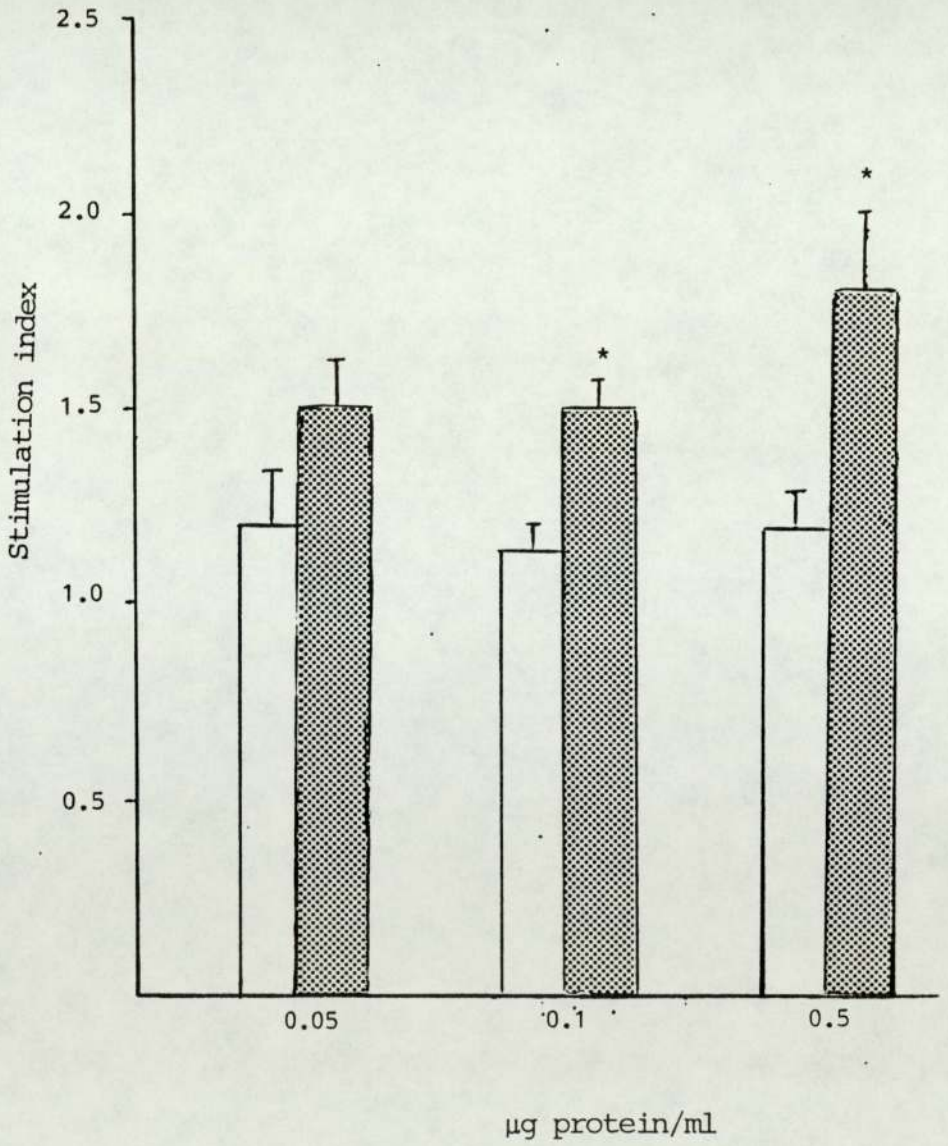


Figure 6.5

Proliferation of lymphocytes from MSZ-treated mice (shaded bar) and control mice (open bar) by mitomycin treated HIT-T15 cells
* $P < 0.05$, n=5

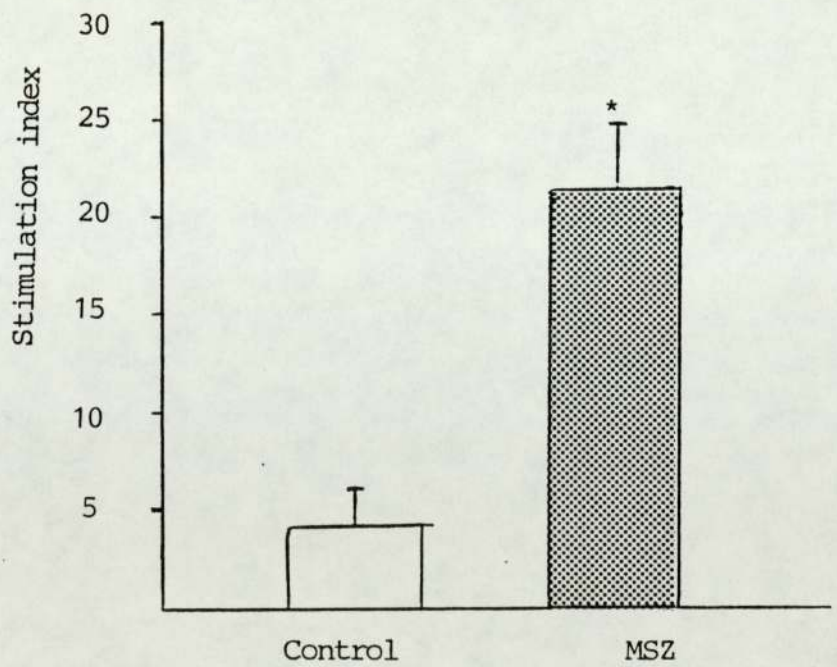


Figure 6.6 Islet cell surface antibodies in the serum of MSZ treated mice against RINm5F cells. (X1000)

6.4 DISCUSSION

Work presented in this thesis has, in part, attempted to demonstrate the existence of cell mediated and humoral immunity against the β-cell in MSZ treated mice. Data to suggest the involvement of autoimmune mechanisms against β-cells in man, BB rat and NOD mice, is beginning to emerge, however, studies on autoimmune aspects of diabetes in MSZ treated mice are scarce (Kruschel et al 1984; Pozzilli et al 1984; Maruyama et al 1984; McKay et al 1985)

In the present study, leucocytic infiltration of islets was detected following MSZ treatment. This observation confirms the original findings of Like and Rossini (1976) and the findings of other researchers (Like et al 1978; Kim and Steinberg 1984). The fact that insulitis was associated with hyperglycaemia suggests that insulitis may have an important role to play in inducing diabetes in MSZ treated mice. However, the mere presence of cells of the immune system in the vicinity of already damaged β cells does not imply that these immune cells are actively involved in the destruction of β-cells. Lymphocytes may be reacting to damaged cells or altered self components on diseased β-cells. This possibility cannot be discounted in the present study since animals were only examined for insulitis after the onset of hyperglycaemia.

Recently, Cossel et al (1985) found that the infiltrating cells appeared only transiently in the islets during the induction of diabetes in MSZ treated mice. Furthermore, single inflammatory cells have been found in such islets long before the induction of diabetes (Kolb-Bachoffen 1988). These studies, together with the fact that immunosuppression techniques such as anti-lymphocyte serum (ALS),azathioprine, irradiation or

thymectomy prevent the induction of diabetes, are suggestive of a role for cell mediated autoimmune reactions in MSZ treated mice (Rossini et al 1978b; Nedergaard et al 1983;Kolb 1987). The nature of the cells involved in the proposed cytolysis for the β-cells is unknown. On the one hand, Cossel et al (1985) found NK cells, but not T cells, in the islets of MSZ treated mice. On the other hand, T-cell depletion was shown to prevent diabetes (Weber et al 1986). Furthermore, when antiserum against Ia antigens, was injected into mice; MSZ induced diabetes was prevented (Kiesel and Kolb 1983; Herald et al 1987; Bonnevie-Nielsen and Lernmark 1986 for a review see Kolb 1987). Normally, however, these Ia antigens are not found on resting T-cells or on β-cells (Faustman et al 1980), but in IDDM, Ia bearing cells were increased (Jackson et al 1982). It is possible therefore, that both NK and T cells are involved in causing cytolysis of the β-cells.

Activation of T-lymphocytes may occur either as a result of an immune stimulation by the β-cell and/or a defect in the immuno-regulation of T-cells (Lohmann et al 1986). Following MSZ treatment, Kay et al (1988) reported that MHC Class II antigens could be detected on the β-cells. This may, under some circumstances, promote T cell help and autoimmunity. The same authors demonstrated that insulitis and hyperglycaemia were associated with aberrant expression of Ia. Furthermore, when islets were cultured in the presence of IL-2 (a T cell growth factor) the amount of insulin secretion was lower than the amount of insulin secretion in the absence of IL-2, or when normal islets were cultured in the presence of IL-2 alone. This result suggested that lymphocytes, already present in the islet, proliferated in-vitro in the presence of IL-2 and consequently lysed β-cells. It is possible therefore, that similar

mechanisms might be involved in-vivo.

Although the mechanisms responsible for the in-vivo induction of MHC expression by the β-cell are unclear, it is known that interferon (released from virus infected cells, and T-cells) can induce DR or Ia antigen expression on human β-cells (Foulis and Farquharson 1986). Certain viruses have been shown to alter MHC expression on human β-cell and RINm5F cells (Campbell et al 1988). Similarly, type C virus might induce aberrant or altered MHC expression in MSZ treated mice. It has been proposed that the aberrant expression of MHC antigens on the virally infected β-cell might induce the presentation of surface autoantigens to T_H cells and the subsequent induction of cytotoxic effector Tc lymphocytes against β-cells (Bottazzo 1984).

In the present study, lymphocytes isolated on the 10th day following MSZ treatment were found to be cytotoxic towards ^{51}Cr-labelled ob/ob mouse islet cells. These results suggested that cytotoxic T cells may have been induced by MSZ treatment. Our findings are consistent with a recent report which found cytotoxic lymphocytes isolated from MSZ treated mice, displayed activity against RIN cells (McEvoy et al 1984 and 1987). Cytotoxic lymphocytes directed against β cells have also been reported in the BB rat (McKay et al 1985) and NOD mice (Maruyama et al 1984). Similarly, lymphocytes isolated from recently diagnosed IDDM patients were shown to be cytotoxic towards rat islet cells (Kruschel et al 1984; Pozzilli et al 1984). To investigate the possible involvement of these cytotoxic lymphocytes in IDDM, McEvoy et al (1984) found that peak cytotoxicity occurred 8-10 days after the first SZ injection, and very little cytotoxicity could be found after 42 days. The transient nature

of the cytotoxicity observed by the above authors, and the fact that in the present study it was detected before the induction of overt diabetes, suggests that the cytotoxic cells may have a pathogenic role in the induction of diabetes. Further support for this proposal comes from the fact that in-vivo studies presented here, have indicated the presence of infiltrating cells in the islets.

Following a 4 hour co-culture of HIT-T15 cells and lymphocytes, the amount of insulin, secreted during a subsequent 90 minute incubation, was found to be significantly lower when lymphocytes from MSZ treated mice were used in the initial co-culture. Co-culture with control lymphocytes, however, did not alter the amount of insulin secreted in the medium, which was comparable to the amount released from HIT-T15 cells cultured alone. Our findings are in accordance with previous studies which utilised lymphocytes from IDDM patients and rat islet target cells (Boitard et al 1981; Lohmann et al 1986).

In studies which measured the amount of insulin in the medium immediately after co-culture of β-cells with isolated lymphocytes from IDDM patients, Lohmann et al (1986) reported that the amount of insulin secreted was significantly greater than the amount of insulin detected after co-culture with control lymphocytes. In contrast, Boitard et al (1981) and Lampeter et al (1984) reported no increase in the amount of insulin released following co-culture of lymphocytes from IDDM patient with β-cells. Lampeter et al (1984) also demonstrated morphologically that granulocytes, present in the lymphocyte population, phagocytosed insulin secretory granules which had been released from lysed β-cells. The authors concluded that during the co-culture of lymphocytes from IDDM patients with rat islets, lymphocytes lysed β-cells, which

resulted in the presence of secretory granules in the medium. However, the secretory granules were phagocytosed and an increase in the insulin content could not be established. It is a possibility therefore, that according to the method used in the preparation of lymphocytes from IDDM patients for the co-culture with islet cells, two types of results can be obtained. Thus, if phagocytes are present, there will be no increase in the amount of insulin, but if the lymphocytic preparation is relatively pure there will be an increase. Indeed, the methods for lymphocyte preparation employed by Boitard et al and Lohmann et al were different. In preliminary studies in this laboratory we found that splenocytes contaminated by phagocytic cells from MSZ treated mice also reduced the amount of insulin secreted from HIT-T15 cells.

Work presented here has shown that splenocytes from MSZ treated mice, when co-cultured with either a homogenate of HIT-T15 cells, or mytomycin-C-treated whole HIT-T15 cells, proliferated more than the control splenocytes. Recently, Itoh et al (1984) found that when lymphocytes from MSZ treated mice were cultured with a homogenate of rat islets, proliferation was raised, but not significantly higher than that of control splenocytes. It is likely that the lack of proliferation in the above study was due to the limited amount of β-cells in the islets. The exact nature of the proliferating cells is unknown, however, T cells are thought to be involved (Bradley 1980). Since the culture period is not long enough to activate virgin lymphocytes, it is presumed that the lymphocytes have been pre-sensitised in-vivo by antigenic determinants on the β-cell and, following in-vitro culture of these lymphocytes with a further antigenic challenge, the lymphocytes proliferate. Similar studies in BB rats have shown that pancreas derived T-lymphocytes proliferated in-vitro in the presence of islet cell

antigen (Scott et al 1982; Prudhomme et al 1984).

The convenience of the availability of HIT-T15 cells and their successful use in the demonstration of cell mediated immunity in MSZ treated mice, therefore, was an important factor in the present studies. Nevertheless, the fact that mouse lymphocytes were able to mount cellular reactions against xenogenic (HIT-T15) or allogeneic (obese mouse islet) cells possessing MHC incompatible antigens, with respect to the effector cells, poses further problems, because effector T-cells may recognise the foreign MHC antigens on the target cell rather than viral or SZ-modified antigens. Most researchers in the past have paid little attention to this, especially since fresh human β-cells are difficult to acquire. Consequently, much research has relied on the use of rat islet cells. It is important in future studies, therefore, to determine to what extent such reactions against the β-cell observed in MSZ treated mice or in IDDM, are a function of virally or MSZ induced changes, or simply reactions against allogeneic or xenogeneic MHC antigens.

In the present study it emerged that serum collected from MSZ treated mice was able to bind to RINm5F cells. This finding revealed that islet cell surface antibodies (ICSA) had been induced in the MSZ treated mice. This observation is in agreement with a recent report which also found ICSA in MSZ treated mice (Itoh et al 1984). This was a significant finding because ICSA have been found in the BB rat and NOD mice (Dyrberg et al 1982; Takei et al 1986) and are thought to play a critical role in the induction of diabetes. It is, therefore, possible that ICSA may also play a similar role in the induction of diabetes in MSZ treated mice.

Whilst it is well known that ICSA are associated with IDDM (Lernmark et al 1978a), the mechanisms involved in the induction of ICSA and their role in the development of diabetes is unclear. It could be that there is an abnormality of the immunoregulatory system which leads the B-lymphocyte to produce ICSA. Normally the B-lymphocyte is under the immunoregulatory influence of suppressor T cells and helper T cells. Any defect in the suppressor T cells, therefore, could lead the B-lymphocytes to start producing autoantibodies. Recently, it was reported that anti-lymphocyte antibodies (ALA) were found in Type I diabetes (Ozturk and Terasaki, 1979) and that there was a correlation between ICSA and ALA in the BB rat (Dyrberg et al 1982). Recent studies have found that 55% of newly diagnosed IDDM were positive for ALA, but only 15% of IDDM of longer duration had ALA (Serjeantson et al 1981). This suggested that ALA may have a pathogenic role in the induction of IDDM. The detection of ALA in other well established autoimmune disorders, such as systemic lupus erythematosis (SLE), supports the above proposal (Morimoto et al 1980). Furthermore, ALA from SLE were shown to react with suppressor T cells, suggesting that ALA may be specific for Ts cells (Korke et al 1979). Certainly, a reduction in suppressor T cells has been reported in IDDM (Slater et al 1980; Buschard et al 1980). It might follow therefore, that certain environmental, genetic or toxic substances (such as SZ) may induce the production of ALA, the subsequent impairment of the suppressive role of Ts cells on the B-lymphocytes may induce the latter to produce antibodies such as ICSA. Recent studies have shown that administration of LPS into MSZ treated mice (Flechner et al 1986), or complete Freund's adjuvant (CFA) into MSZ treated rats (Ziegler et al 1984), enhanced the diabetes. Both LPS and CFA have also been shown to be polyclonal B lymphocyte activators (Huber et al 1976) and may induce an even higher level of ICSA, thereby exacerbating the induction of diabetes.

The exact mechanisms involved in the induction of ICSA and their possible significance in the pathogenesis of diabetes in MSZ treated mice are undefined, SSZ treatment causes diabetes by direct toxicity on the β-cell (Nichols et al 1978). In a preliminary study we found that SZ was cytotoxic to cultured HIT-T15 cells. MSZ treatment also causes direct β-cell loss (Bonnevie-Nielsen 1981), however, this is insufficient by itself to cause diabetes, since various immunosuppressive means can block the induction of diabetes (Kiesel and Kolb 1983). It is possible therefore, that the initial toxicity of MSZ treatment may release previously sheltered antigens or that SZ modifies the proteins present on the β-cell, thereby rendering them antigenic to the host immune system. Islet cell antibodies are not peculiar to mice following MSZ treatment, since they have also been detected in humans who had attempted suicide with the rodenticide Vacor ®, whose active component has a structure and physical activity similar to that of SZ (Karam et al 1980). The pathological effect(s) of ICSA in causing β-cell toxicity may be brought about in several ways. In the presence of complement, ICSA from IDDM can lyse β-cells (Dobersen et al 1980; Kanatsuma et al 1982). Furthermore, ICSA in conjunction with Fc bearing K cells (ADCC cells) can also lyse β cells (Kohler et al 1984).

It is a possibility, therefore, that ICSA in MSZ treated mice may have a similar pathogenic role in inducing diabetes, especially since it was previously shown that ADCC was increased following MSZ treatment (Chapter 5). ICSA from patients with IDDM were found to react against a 64 KD integral plasma membrane protein present on β cells (Baekkeskov et al 1982). If it can be shown that ICSA from MSZ treated mice might also be sensitive to a similar protein on the β-cell, then this would lend

great support to the involvement of humoral immunity in the pathogenesis of diabetes in this model.

In contrast, however, the influence of ICSA on β-cell function in-vitro does not necessarily mean that these cells are affected under in-vivo conditions. Although ICSA may circulate at high levels, it does not follow that they can cross the capillary barrier to reach the target antigen on the β-cell surface. Svennigsen et al (1983) attempted to provide direct proof for the involvement of ICSA in diabetogenesis by injecting ICSA prepared from the plasma of Type I diabetic children into mice. Glucose induced insulin release was inhibited but the total pancreatic insulin content was unaffected. This in-vivo study suggests that ICSA may be insufficient by itself to cause sufficient β-cell destruction to result in IDDM. Further studies are required to evaluate the pathogenic role (if any) of ICSA.

In summary, these data provide evidence for the hypothesis that hyperglycaemia in mice following MSZ treatment may be due, at least in part, to activated autoimmune responses against the β-cell. Both humoral and cell mediated immune responses are likely to be involved. ICSA could be cytotoxic towards β cells by fixing complement or via K cell, and cytotoxic T cells might also be involved. For absolute proof of the involvement of autoimmune reactions in MSZ-treated mice, however, further studies are required (e.g. passive transfer of diabetes by T-cells from MSZ-treated mice into normal mice and/or the prevention of MSZ-induced diabetes by immunosuppression).

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APPENDIX

A1 Citrate Buffer

Citrate buffer was prepared by mixing 22ml of 0.1 Mol/l sodium citrate and 28m 0.1 Mol/l citric acid, distilled water was added to make 100ml and the pH was adjusted to 4.2

A2 Basic Krebs / Bicarbonate / HEPES Incubation Buffer

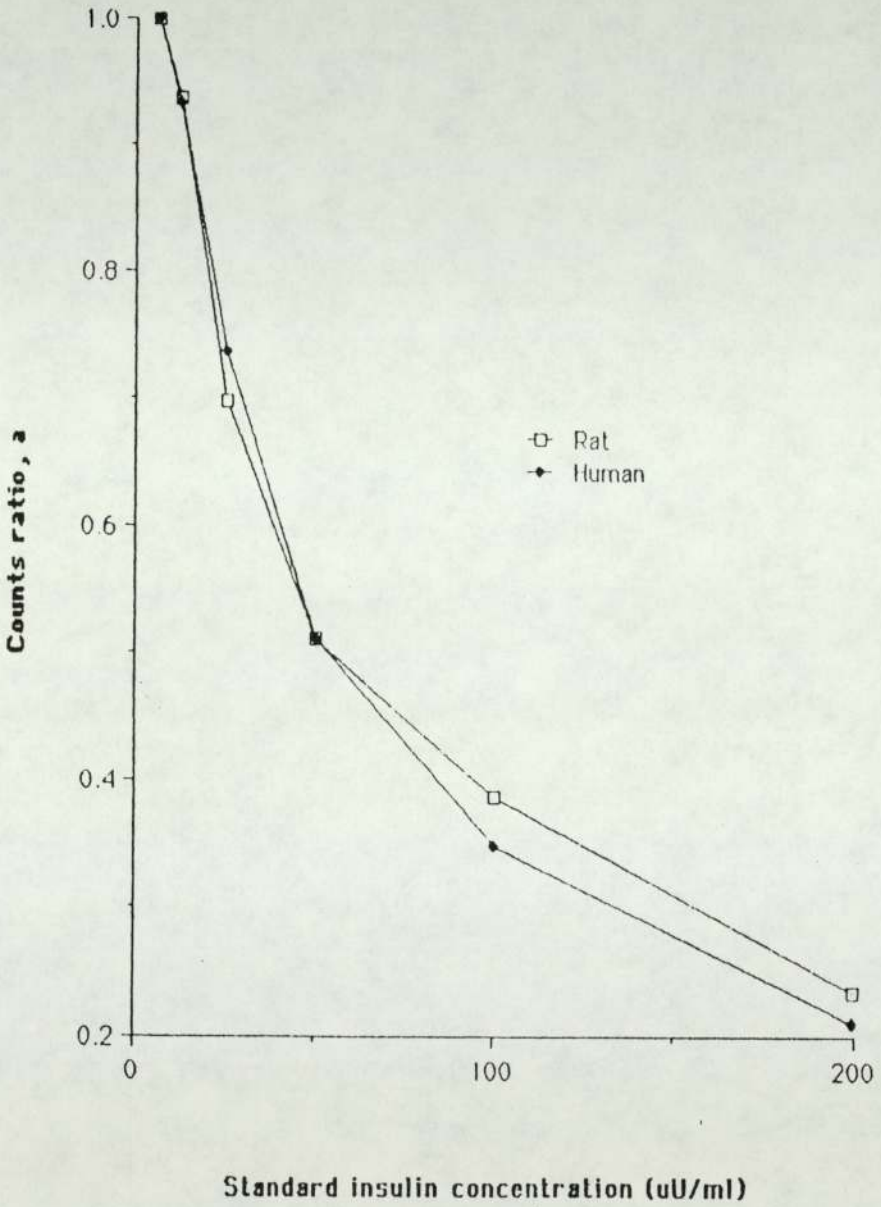
The incubation medium used throughout this thesis was krebs / bicarbonate / HEPES with the following composition, molarity (μMol/l) is shown in parentheses.

NaH_2PO_4 (0.2), $NaCO_3$ (20), Na_2HPO_4 (1.8), KCl (4.5), $MgSO_4$ (1.2) and $CaCl_2$ (2.56).

After gassing the buffer for 20-30 minutes with 95% O_2 : 5% CO_2, HEPES (16) was added and the pH adjusted to 7.4 with either 2 Mol/l NaOH or 2 Mol/l HCl.

A3 Comparison of standard curves constructed using rat and human insulin standards in the double antibody radioimmunoassay

Data are means of 3 separate assays .For clarity SEM's are not shown but in all cases were < 8 %,$p > 0.05$, no difference between standards at all concentrations.



a counts in the bound fraction for each standard were expressed as a ratio of the 6.25 *u*U/ml standard .This moneouvre reduces the effect of slightly differing amounts of total lable added in diffent assays.