THE UNIVERSITY OF CALGARY

STUDIES ON THE ROLE OF CYTOTOXIC ISLET CELL SURFACE AUTOANTIBODIES IN THE PATHOGENESIS OF INSULIN-DEPENDENT DIABETES MELLITUS

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Studies on the Role of Cytotoxic Islet Cell Surface Autoantibodies in the Pathogenesis of Insulin-Dependent Diabetes Mellitus" submitted by Karen E. Tanguay in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

Insulin-dependent diabetes mellitus (IDDM) results from the destruction of the beta cells within the pancreatic islets of Langerhans. Environmental factors, genetic susceptibility and autoimmune mechanisms are implicated in the pathogenesis of the disease, however, the precise etiology remains unclear. Extensive evidence suggests that, in a large number of patients, the beta cell damage is caused by an autoimmune process. This process is possibly triggered by exogenous factors, such as a virus, in genetically susceptible individuals.

The possibility that humoral autoimmune mechanisms are involved in the development of IDDM is suggested by the detection of antibodies to the cytoplasm and the surface of islet cells in the sera of patients with IDDM. The group of autoantibodies directed against the surface membrane of beta cells seems to most closely correlate with the pathogenesis of the disease. These antibodies are often present in the sera of both human insulin-dependent diabetics and spontaneously diabetic biobreeding (BB) rats. The BB rat provides an excellent animal model for IDDM as it exhibits many similarities to the human disease.

Although the islet cell-surface autoantibodies (ICSA) have been implicated in the disease process, whether a correlation exists between ICSA and diabetes remains controversial. In this study the presence of cytotoxic ICSA was examined in 65 BB rats from 60 to 150 days of age. The results showed that while 29% of the rats exhibited cytotoxic ICSA

and diabetes, 22% were overtly diabetic without showing any measurable ICSA. An additional 22% showed ICSA but did not become diabetic in the time period studied. Furthermore, induction of ICSA, by injecting prediabetic BB rats with rat insulinoma cells, did not cause diabetes. These results suggest a lack of correlation between cytotoxic ICSA and overt diabetes in our BB rat colony. Moreover, extensive analysis of the data implies that the expression of ICSA may be heritable and segregation of the determinants for ICSA production and diabetes may be possible.

Although, from the present findings, ICSA do not appear to be primary mediators of the disease they may have predictive value as markers for IDDM. Therefore, to study the role of cytotoxic ICSA, a monoclonal autoantibody, designated KT1, was produced from the fusion of spleen cells from a BB rat and a mouse myeloma cell line. This monoclonal antibody was found to be of the immunoglobulin M isotype and reacted specifically with islet cells. In microcytotoxicity assays KT1 mediated complement-dependent lysis of approximately 30% of rat insulinoma cells and approximately 40% of rat pancreatic islet cells, but did not cause lysis of the other cell lines tested. KT1 binds specifically to a cell surface antigen on live and fixed islet cells from normal rat pancreas and to rat insulinoma cells by indirect immunofluorescence. In western blotting experiments, KT1 reacts with a 68,000 dalton protein from rat insulinoma cells. This monoclonal antibody may provide a useful tool to investigate the clinical aspects of IDDM as it exhibits properties similar to the ICSA present in the sera of BB rats.

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To my husband Richard and

our daughter Elyse.

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ABBREVIATIONS

A cells	alpha cells
ADCC	antibody-dependent cell-mediated cytotoxicity
AMLR	autologous mixed lymphocyte reaction
BB rat	biobreeding rat
B cells	beta cells
BHK	baby hamster kidney
BSA	bovine serum albumin
CF-ICA	complement-fixing islet cell antibodies
CHAPS	(3-[3-cholamidopropyl)dimethyl ammonio]' propane sulfonate
d	dalton
D cells	delta cells
DMEM	Dulbecco's minimum essential medium
FCS	fetal calf serum
FITC	fluoroscein isothiocyanate
HLA	human leukocyte antigens
ICA	islet cell antibodies
ICSA	islet cell surface autoantibodies
IDDM	insulin-dependent diabetes mellitus
Ig	immunoglobulin
IL-2	interleukin 2
Ir	immune response
-kD	kilodalton
Mab	monoclonal antibody

MHC	major histocompatibility complex
MKD	monkey kidney
NC	nitrocellulose
NIDDM	non-insulin-dependent diabetes mellitus
NOD	'non-obese diabetes'
PBS	phosphate buffered saline
PEG	polyethyleneglycol
pp	pancreatic polypeptide
SD	standard deviation
SLE	systemic lupus erythematosus
TRITC	tetramethylrhodamine isothiocyanate

INTRODUCTION

The precise etiology of human insulin-dependent diabetes mellitus (IDDM) is not known. It is likely to depend on an interplay between inherited traits and environmental factors. Moreover, there is rapidly expanding evidence which suggests that IDDM is an autoimmune disease. This data has come from studies of morphology, cellular immunity, and humoral immunity, as well as from clinical trials of immunosuppressive therapy. The opening chapter of this thesis describes the pathogenesis of the disease encompassing the genetic and environmental elements which may be involved. A large proportion of this chapter will discuss the relevance of autoimmunity in IDDM focusing on islet cell surface antibodies which are the subject of this thesis.

Over the past decade, a number of investigators have reported that sera from patients with IDDM contain antibodies which react with pancreatic islet cells (Doniach and Bottazzo, 1977; Lernmark et al., 1978; Doberson et al., 1980a). Among these, a group of antibodies specific for a beta cell membrane antigen(s) appear to be most closely associated with the pathogenesis of the disease. These autoantibodies are specifically cytotoxic to beta cells (Doberson et al., 1980a; Kanatsuna et al., 1981) and have been shown to inhibit the in vitro release of insulin. Because of these and other interesting features islet cell surface autoantibodies (ICSA) have been implicated in the disease process. However, no conclusive data are available on whether there is a correlation between the presence of cytotoxic ICSA and the onset of diabetes.

The frequency of ICSA in patients with IDDM varies considerably between studies (32-94%) (Lernmark et al., 1978; Van De Winkel et al., 1982). These antibodies are also found in a significant proportion of first degree relatives (Doberson et al., 1980a). Therefore, it is not yet clear if these autoantibodies play a primary role in the destruction of beta cells or if they are a secondary phenomenon associated with the onset of IDDM but not causing the disease. It is difficult to isolate and study the relative importance of ICSA in the etiology of IDDM in humans. It would be necessary to monitor levels of ICSA in prediabetics but these individuals are not readily identifiable. For this reason, it is more practical to employ animal models in which to evaluate the possible role of ICSA in causing diabetes.

The spontaneously diabetic Biobreeding (BB) rat presents a syndrome that is very similar to the disease process in man and, therefore, provides an excellent animal model of IDDM (Nakhooda et al., 1978). The relevant characteristics of the BB rat will be discussed in detail later. ICSA have also been identified in the sera of BB rats (Dyrberg et al., 1982) however, their role in the pathogenesis of the disease is unclear (Dyrberg, 1986). It has previously been reported that antibody-dependent cytolytic activity against islet cells can precede the manifestation of overt diabetes by several weeks in BB rats (Martin and Logothetopoulos, 1984). However, whether or not a correlation exists between ICSA and diabetes in these animals remains controversial.

The aim of this research project was to examine the association between the presence of cytotoxic ICSA and the development of overt diabetes in BB rats, in order to determine if a correlation exists. In

addition, whether or not ICSA prove to be primary mediators of immune damage in IDDM it remains likely that they contribute to the destruction of the beta cells. Therefore, it would be of value to analyse ICSA in vitro. This could be accomplished by cloning these autoantibodies using hybridoma techniques in order to produce a large quantity of identical antibodies. These could be used not only to investigate the role of ICSA in the pathogenesis of IDDM but also to characterize the autoantigen to which these antibodies are directed.

I. CURRENT STATE OF KNOWLEDGE

A. Diabetes Mellitus in Humans

Diabetes mellitus is a chronic, metabolic disorder which adversely affects the body's ability to synthesize and/or utilize insulin, a hormone required for glucose metabolism. It is a very ancient disease, the earliest description of its symptoms dating back to 1500 B.C. Polyuria, polydipsia, polyphagia and mellituria have long been recognized as the classic symptoms of diabetes mellitus. Modern research has shown, however, that diabetes is a heterogeneous group of disorders which have hyperglycemia in common. In addition to its effects on carbohydrate metabolism, diabetes also affects the metabolism of protein and fat and commonly leads to serious complications affecting many organs (Notkins, 1979).

Diabetes mellitus ranks among the top ten causes of death in Western nations, and despite many improvements in its clinical management, it has not been possible to significantly control its lethal consequences. In its various forms, diabetes afflicts about five percent of the population in most Western societies (Notkins, 1979). Studies of the natural history and pathogenesis of hyperglycemia together with recent advances in biotechnology, have led to the system of classification developed by the National Diabetes Data Group (1979). Among the various diabetic syndromes now recognized, insulin-dependent (Type 1 or juvenile onset) and non-insulin-dependent (Type II or maturity onset) diabetes mellitus account for a great majority (> 90%) of patients with the disease.

1. Insulin-Dependent Diabetes Mellitus

Insulin-dependent diabetes mellitus (IDDM) is the severe form of the disease. Although it can appear at any age, it most commonly occurs during adolescence, with the peak age of onset being 12-13 years. IDDM is the most prevalent chronic childhood disease affecting approximately one individual in a thousand. Before the discovery of insulin by Banting and Best in 1921, children died within 2-3 years after onset of the disease (Banting and Best, 1922). Although the prognosis for children with IDDM has rapidly improved, the long term complications still result in considerable morbidity, often in the early adult years. Those afflicted with the disease develop a life-long dependence on insulin injections for survival. This therapy now prevents death from diabetic ketoacidosis that was formerly inevitable, but insulin therapy does not prevent the late complications of IDDM. These include blindness, renal failure, neuropathy and peripheral and coronary vascular disease (Rossini and Chick, 1980). Although studies on immune intervention of diabetes with immunosuppressive agents such as cyclosporin A are presently at the phase of controlled clinical trials (Stiller et al., 1984), the only accepted treatment of the disease to date is a combination of insulin therapy, diet and exercise. At present, there is no accepted, safe way in which to prevent the disease or to alter its course once it has begun.

2. Morphology and Function of the Normal Endocrine Pancreas

It may be helpful to begin this description of the pathogenesis of IDDM with a brief overview on the normal pancreas. A comprehensive review of this topic is provided by Robbins (1984). The endocrine

pancreas consists of about 1 million microscopic cellular units, called the islets of Langerhans, and a few scattered cells within the small pancreatic ducts. In the human adult, islets measure from 50 to 250 um and consist of four cell types: B (beta), A (alpha), D (delta) and pp (pancreatic polypeptide) cells. The beta cell is of major importance to this thesis because of its relevance to diabetes mellitus and will be described more fully later. Alpha cells secrete glucagon, which can induce hyperglycemia by its glycogenolytic activity in the liver. They make up about 20% of the islet. Delta cells, which make up 5-10% of the islet, contain somatostatin, a hormone which suppresses both insulin and glucagon release. Only 1 to 2% of the islet is composed of pp cells. These cells contain a unique pancreatic polypeptide that exerts a number of gastrointestinal effects when injected into experimental animals, but its physiologic significance in humans is unknown.

Beta (B) cells make up about 70% of the islet and have been studied the most intensely because of their ability to synthesize and secrete insulin. Insulin is produced in the beta cell by enzymatic cleavage of the precursor polypeptide called proinsulin. It is then stored within membrane-bound cytoplasmic granules derived from the Golgi apparatus. The granules have rectangular profiles and a crystalline matrix. The primary stimulus for insulin secretion is glucose, although amino acids such as arginine and leucine are also potent insulin secretagogues.

Receptors for insulin occur on a variety of cells including fat cells, monocytes, red blood cells and fibroblasts. Insulin is involved in many important cell processes such as the enhancement of membrane transport of glucose, amino acids and certain ions. This hormone also

induces the cellular storage of glycogen, formation of triglycerides and synthesis of protein, RNA and DNA. However, the mechanism by which insulin controls all these processes has yet to be determined.

3. Pathogenesis of IDDM

IDDM results from the destruction of the beta cells in the islets of Langerhans, leading to an absolute or severe lack of insulin. In the fully expressed condition, the pancreas contains little or no extractable insulin and has an overall reduction in beta cell mass (Lacy, 1977). Plasma insulin levels are low and respond poorly, if at all, to the administration of glucose or other stimulators of insulin secretion. Therefore, patients with IDDM require exogenous insulin administration to prevent the ketoacidosis and death, hence the term "insulin-dependent". Although the insulin deficiency is established, the etiology of beta-cell injury is still unclear. Environmental factors, genetic susceptibility and autoimmune mechanisms have all been implicated in the pathogenesis of the disease. On the basis of the available genetic data (particularly studies on twins) it is obvious that environmental factors must be involved. Although no definite environmental agent has yet been identified viruses are considered to be the prime suspects (Notkins, 1979).

4. Environmental Factors and IDDM

A number of etiological factors have been suggested to contribute to the pathogenesis of IDDM. Viruses, stress, environmental toxins and various immune system alterations have all been proposed. It seems likely that there is no single cause of IDDM but a variety of genetic

and environmental causes. In some cases insults from the environment such as drugs and toxic chemicals may damage beta cells and give rise to diabetes (reviewed in Yoon et al., 1987a). In this connection the drug alloxan has been shown to selectively destroy beta cells in experimental animals (McLetchie, 1982). Another drug, streptozotocin is directly toxic to the beta cells of experimental animals if given in a single large dose (Cooperstein and Watkins, 1981). On the other hand, multiple small doses of streptozotocin administered to susceptible mice appear to act indirectly, presumably by altering the beta cells so that they become vulnerable to attack by the animals own immune system (Like and Rossini, 1976). These mice develop antibodies against their pancreatic islet cells. Under the latter conditions only certain strains of mice develop diabetes, again suggesting the importance of genetic factors. A number of people have become diabetic after ingestion of the beta cytotoxic drug Vacor (a rodenticide) with a molecular structure similar to streptozotocin. These individuals were also found to exhibit islet cell antibodies (Karam et al., 1978). It is possible that among the thousands of chemicals that humans are exposed to daily, there may be some which are able to trigger the pathway which can eventually result in beta cell damage and IDDM. On the other hand a large body of evidence indicates that loss of beta cells may be initiated by viral infection of the islets. This aspect of IDDM will be discussed in the following section.

a. Viruses and IDDM

For many years there have been sporadic reports of a close

temporal association between common childhood viral infections such as mumps, measles, influenza and Coxsackie B infections and the onset of IDDM (Craighead, 1975). Seasonal variations in the diagnosis of new cases of IDDM corresponding to the prevalence of these viral infections have also been noted. In one of these studies, patients with IDDM of recent onset were found to have high titers of neutralizing antibody to Coxsackie B4 virus when compared either with normal subjects or with patients with longterm diabetes (Gamble et al., 1969a). In addition, Gamble and Taylor (1969b) demonstrated that the seasonal incidence of diabetes correlated with the annual prevalence of Coxsackie virus infection. A decade later, Yoon and coworkers (1979) provided the most convincing evidence of a possible relationship between viral infection and IDDM. They isolated Coxsackie B4 virus from the pancreas of a child who died of diabetic ketoacidosis after a flu-like illness and added new momentum to the field. Inoculation of mice with the human isolate of the Coxsackie B4 virus led to hyperglycemia, inflammatory cells in the islets of Langerhans and beta cell necrosis. Immunofluorescent staining revealed that viral antigens were present in the beta cells providing strong support for a direct viral etiology for IDDM (Yoon et al., 1979). Moreover, patas monkeys infected with Coxsackie B4 virus showed a transient elevation of glucose tolerance tests, depressed insulin secretion and glucose in the urine. No islet cell antibody activity was found in their sera (Yoon et al., 1986). Thus, it has been suggested that a group of viruses may directly destroy beta cells thus inducing IDDM independent of autoimmune processes (Yoon et al., 1987b).

IDDM has been reported in up to 20% of patients with congenital rubella (Forrest, 1971) and experimental rubella infection in rabbits was shown to cause morphological changes in the pancreas (Menser, 1978). Moreover, encephalomyocarditis virus-neutralizing antibody was found in 12% of patients with IDDM compared to 6% of a control population (Yoon, 1977). Craighead (1981) demonstrated that a diabetes-like disorder can be induced in certain strains of mice by subcutaneous inoculation of the M-variant of encephalomyocarditis virus. This microbiological agent appears to localize specifically within the islets of Langerhans to produce direct viral damage to the beta cells. Thus, there is evidence to support the notion that viral infections may injure beta cells in humans and animal 'models'. However, overwhelming virus infection with direct beta cell injury is clinically atypical of IDDM and probably contributes to only a fraction of cases. More often a history of virus infection, if present, relates to a relatively minor illness months before the detection of diabetes. Therefore, the exact role of viruses in the pathogenesis of human IDDM is not known. One theory proposes that the beta cell mass may be gradually eroded by autoimmune reactions of unknown origin and a subsequent viral infection may simply cause the final critical reduction in beta cell numbers which results in the manifestation of overt diabetes (Srikanta, 1983a).

It has also been suggested that hidden antigenic determinants on the beta cell surface are exposed by viruses, or other pathological agents, and become the target for autoimmune attack (Uchigata et al, 1987). However, data from a number of studies, which shall be discussed later, dispute this theory. An alternate hypothesis is suggested by the observation that a transient production of islet cell

antibodies in children occurred during the first year after mumps infection (Helmke et al., 1980). Further evidence was provided by Onodera et al. (1982) who demonstrated that islet specific autoantibodies occurred after reovirus infection of mice. This was supported by a recent study showing that autoantibodies to various endocrine target tissue can be induced by injecting neonatal animals with reovirus (Haspel, 1983). Thus, although autoimmunity may develop in genetically susceptible individuals in response to a viral trigger, the precise mechanism remains to be determined. Therefore, while viruses may be involved in the pathogenesis of some cases of IDDM, additional studies are required to determine the relative importance of viruses in the disease process.

5. Genetics of IDDM

Another major contribution to understanding the pathogenesis of IDDM has come from studies on the role of hereditary factors in the disease. IDDM is a heritable disorder, but the exact mode of transmission is unknown (Rimoin et al., 1984). Family studies indicate clearly that relatives of probands with IDDM become diabetic with much greater frequency than do people in the general population (Soeldner, 1982). If one parent has IDDM, the risk is about 8-10% for their offspring to develop the disease. If both parents have IDDM, the risk is over 23%. The total genetic contribution does not appear to exceed 50% since this is the maximum estimate of concordance for identical twins to both contract the disease.

Almost all modes of inheritance have been suggested, but the evidence does not conclusively support any one hypothesis. A

single-gene model would not account for the heterogeneity of IDDM, and various polygenetic models have been proposed (Rotter, 1981, Thomson, 1980). The most powerful insights into the genetics of IDDM have come from the study of the genes which code for the histocompatability antigens. The association between IDDM and certain human leukocyte antigens (HLA) has been known for over a decade (Cudworth and Woodrow, 1975). HLA, the major histocompatability region in man, refers to a linked set of genes located on the short arm of chromosome 6. Molecules encoded by these genes are present on cell surfaces and play an important role in the immune response. HLA class I antigens are present on virtually all nucleated cells in the body and are involved in immunologic rejection of foreign cells, whereas the class II genes are more restricted in their distribution and are important in the control of the immune response. Initially the genetic linkage in IDDM was thought to be with class I major histocompatability complex (MHC) genes. Unlike other associations between HLA and disease which generally involve only a single antigen, IDDM presents a more complex relationship. Combined data on HLA-A, -B and -C antigen frequencies in Caucasian diabetics indicate that the antigens most frequently found in positive association with IDDM are HLA-B8 and -B15, followed by -B18, -B40 and -CW3. Conversely, significant negative associations have been found with HLA-B7, -B5 and -BW35 (Nerup, 1978).

Recently, it has become apparent that there is a stronger link with class II MHC genes especially the HLA-DR region (Platz et al., 1981) and the -DQ region (Todd et al., 1987). The HLA class II antigens are heterodimeric (alpha and beta chains) glycoproteins which are normally present only on immunocompetent cells (B lymphocytes and

certain subsets of macrophages and T lymphocytes). These antigens are believed to mediate certain cell-cell interactions which are essential for the function of the immune system. Consequently, this observation has been taken as additional evidence that immunological mechanisms are important in the pathogenesis of IDDM. In view of the fact that several immune response (Ir) genes map within the D region, it has been suggested that the diabetogenic genes may actually be Ir genes that regulate immune reactions against the pancreatic beta cells. There is evidence that the immune responsiveness of an individual may be partly determined by amino acid sequences within the class II molecules (Cairns et al., 1985). Positive associations have been found with -DR3 and -DR4 haplotypes and negative associations with -DW2. More specifically, among young onset patients with IDDM, more than 90% were found to be positive for HLA-DR3 and/or HLA-DR4 haplotypes (Rotter, 1983). Moreover, population studies indicate that the risk of becoming diabetic is 1 to 3% if a subject is HLA-DR3/-DR4 heterozygote (Gorsuch, 1982). Therefore, it could be speculated that genes confirming susceptibility to IDDM are located at or near the HLA-DR locus. However, since 50% of the non-diabetic population also possess the DR3 or DR4 alleles, the story is even more complex.

The HLA-D region has recently been studied in more detail using recombinant DNA technology. cDNA complementary to the HLA-DR and -DQ antigens mRNA has been cloned in bacterial plasmids (Owerbach et al., 1983). These cDNA probes were used in genomic blotting techniques to uncover differences in the size of fragments generated by restriction endonuclease treatment of DNA isolated from diabetic versus nondiabetic individuals. Restriction fragment length polymorphisms have been

discovered in human IDDM and in animal models of IDDM. According to some of the studies on restriction fragment length polymorphisms, HLA-DQ genes are more strongly associated with IDDM than are the -DR genes (Owerbach et al., 1983; Bohme et al., 1986). A very recent report claims that the structure of the -DQ molecules, in particular residue 57 of the beta chain, specifies the autoimmune response against the beta cells (Todd et al., 1987).

However, the frequencies of the various HLA antigens in diabetic patients differ between different populations and there does not appear to be any unique class II sequences found exclusively in IDDM patients. It is possible that the HLA-D antigens themselves do not directly predispose an individual to diabetes but that the susceptibility is due to genes that occur in linkage disequilibrium with the genes coding for the associated HLA antigens.

In conclusion, it may be a long time before the diabetogenic genes and their product(s) are identified. Moreover the mode of inheritance has still to be defined although a multifactorial inheritance is currently favoured since it best fits the genetic analyses and also provides a role for environmental factors.

6. Evidence for an Autoimmune Etiology for IDDM

Over the past decade an extensive amount of evidence has suggested that the damage found in a large number of IDDM patients results from an autoimmune process directed at the insulin producing pancreatic beta cells which ultimately leads to their destruction (Notkins, 1979; Nerup and Lernmark, 1981).

Recent prospective studies on individuals at high risk for IDDM have indicated that IDDM is a chronic, rather than an acute disease. A long euglycemic period, characterized by immune and metabolic abnormalities has been shown to precede the development of overt hyperglycemia by several years. Clinical manifestations of IDDM develop only when a sufficient number of beta cells are destroyed. Although there appears to be a great deal of heterogeneity within IDDM, the majority of patients exhibit at least some autoimmune phenomena (Drell and Notkins, 1987). It has been suggested that viruses or other environmental factors may initiate an immune-mediated destruction of beta cells in genetically susceptible individuals (Yoon, 1983). An autoimmune hypothesis is supported by several findings. These include the lymphocytic infiltration of islets observed early in the course of the disease; abnormalities of cell-mediated and humoral immune function; and the aforementioned characteristic associations between HLA antigens and the disease.

a. Insulitis

Evidence for an autoimmune etiology for IDDM dates back to 1910 with the description of round cell infiltration of the pancreas (Opie, 1910). In 1965, Gepts reported that insulitis, a mononuclear cell infiltration of the islets of Langerhans, was common in patients with newly diagnosed IDDM. It was found in 70-90% of autopsy cases in which patients died within a few months after the onset of IDDM. Immunofluorescent examination of the cells infiltrating the pancreas of a child with recent-onset IDDM showed that most were cytotoxic/suppressor cells and to a lesser degree T-helper cells (Bottazzo, 1983a). Ninety

percent of the infiltrating T-lymphocytes expressed HLA-DR antigens which is an indication of activated T lymphocytes. This description of insulitis is suggestive of a typical immune response to infection or an autoimmune reaction.

b. Immunoregulatory Abnormalities

Studies concerning the enumeration of circulating blood lymphocytes have been conflicting, showing an excess of B lymphocytes (Bersani et al., 1981), a reduction of peripheral T lymphocytes (Selam, 1979; Mascart-Lemone et al., 1982), or a normal lymphocyte population (Pozzilli et al., 1983; Buschard et al., 1983). Possibly these discrepancies could be related to methodology or to the degree of metabolic control of the disease.

Although B lymphocyte numbers are most often reported as being normal (Horita et al., 1982), increased numbers have been found when islet-cell surface antibodies were present in the serum (Bersani et al., 1981). Furthermore, increased immunoglobulin producing B-lymphocytes have been noted in the blood of patients with IDDM, suggesting the presence of activated, antibody-producing B lymphocytes. This will be discussed in more detail later. Many investigators have demonstrated an imbalance of T cell subsets during early pathogenesis of IDDM using monoclonal antibodies specific for T cell surface markers, conjugated to fluorescent dyes. These include the presence of activated DR-positive T cells (Jackson et al., 1982; Pozzilli et al., 1983), the decrease of suppressor cell number and function (Buschard et al., 1980) and an increased number and function of killer cells (Pozzilli et al., 1979).

An increased number of circulating HLA-DR-antigen-bearing T cells were found to be present in recently diagnosed patients, which decrease with the duration of diabetes (Jackson et al., 1982; Pozzilli et al., 1979). The presence of HLA-DR-positive cells generally reflects activation of the immune system. There have been similar findings reported in other autoimmune diseases such as Graves disease and systemic lupus erythematosus (SLE) (Jackson et al., 1982).

Suppressor cell activity has also been studied by a number of research groups. Buschard and coworkers (1980), using a functional assay based on activation of suppressor cells by concanavalin A, found a reduction in suppressor cell activity in IDDM of recent onset. Jaworski and coworkers (1983) showed that a higher proportion of HLA-DR3 positive diabetics had impaired suppressor cell function when compared to HLA-DR3 negative diabetics. Aberrant suppressor cell function has also been reported in several other diseases, which have in common an autoimmune basis and an association with specific alleles at the -DRW locus, including SLE, myasthenia gravis, autoimmune haemolytic anemia and multiple sclerosis. Suppressor cell dysfunction may be necessary for the development of the disease and it is unlikely that it is a result of the hyperglycemia present in IDDM. This is supported by reports that not only is normal suppressor cell activity found in hyperglycemic patients with non-insulin dependent diabetes mellitus (NIDDM) (Fairchild et al., 1982; Pozzilli et al., 1983) but Buschard and coworkers (1982) have shown that the degree of glycemic control in patients with IDDM had no effects on suppressor cell activity. Abnormal suppressor cell function is also found in healthy, first degree relatives of IDDM patients (Jaworski et al., 1983). This

suggests that the decrease is unlikely to be a consequence of IDDM, nor is it a sufficient condition for the development of IDDM. However, this decrease in suppressor cell number and function could possibly contribute to the activation of B cells mentioned previously.

Finally, an increase in the number of K cells, calculated from the number of low affinity erythrocyte-rosette forming cells (assuming they consist of mostly K cells), was found in newly diagnosed diabetic patients (Pozzilli et al., 1979). Both the DR-positive T cell and the K cell abnormalities were also found in 50% of unaffected siblings who were gentically susceptible and were islet cell antibody positive. It is clear, therefore, that many immune abnormalities exist in IDDM and there is incongruity between reports. More work is required to determine what these immune defects signify.

A recent report describes five patients with newly diagnosed IDDM in whom peripheral lymphocytes were isolated, tagged with indium III and reinjected into the same recipient (Kaldany et al., 1982). Three of the five patients who received autologous radiolabeled lymphocytes appeared to show localization of activity in the region of the pancreas, as detected by emission-computerized scanning. This potentially important study suggests that migration of lymphocytes to the islets occurs in IDDM and may be detected noninvasively.

c. Evidence for Cell-Mediated Autoimmunity

Evidence for cell mediated autoimmunity directed against the endocrine pancreas was initially provided by Nerup et al. (1971) with the demonstration that the in vitro migration of leukocytes from patients with IDDM was inhibited by exposure to antigens prepared from

endocrine pancreas. Furthermore subcutaneous injection of porcine pancreatic homogenate in patients who were positive for leukocyte migration inhibition provoked a delayed hypersensitivity skin reaction to the antigen(s). Cell-mediated cytotoxicity of peripheral blood lymphocytes from diabetic patients towards a human insulin-secreting cell line has been demonstrated (Huang and Maclaren, 1976). Insulinoma cell death, measured by eosin uptake, was shown to be significantly increased following incubation with lymphocytes from IDDM patients compared to those from normal individuals. Furthermore the cytotoxicity was shown to be target cell specific. Unfortunately, this human insulinoma cell line is no longer available and the report lacks confirmation. However, a report claiming lysis of ^{51}Cr-labelled pancreatic islets by lymphocytes from patients with IDDM in a microcytotoxicity assay supports a cell-mediated cytotoxic reaction (Scott et al., 1981). Lymphocytes from a patient with acute IDDM passively transferred to athymic mice caused a slight rise of blood glucose in these animals (Buschard et al., 1978); however, other laboratories have been unable to confirm this finding.

Lymphocytes from IDDM patients have been shown to affect the function of beta cells in vitro. When peripheral blood lymphocytes are obtained from IDDM patients and incubated with mouse islets, they reportedly inhibit insulin secretion when the islets are perturbed with either glucose or theophylline (Boitard et al., 1981). A defect of lymphocyte function has been found using the autologous mixed lymphocyte reaction (AMLR) where the proliferative response of T cells to autologous non-T cells is measured by ^{3}H thymidine incorportion (Gupta et al., 1983). A deficient AMLR has been found in patients with IDDM.

A study suggest that the defect in the AMLR may be due to a defect of the responder T cells and stimulator non-T cells as well as a deficiency in interleukin-2 (IL-2) production (Chandy et al., 1984). Further evidence of insufficent IL-2 production in IDDM has been provided by Rodman et al. (1984). Interferon production has also been shown to be deficient in IDDM (Baratono et al., 1980).

Consequently, there is quite a body of evidence implicating cell-mediated autoimmunity as a possible cause of diabetes. In addition, suppressor T cell activity is reportedly defective when lymphocytes from patients are stimulated by pancreatic antigens (Buschard et al., 1980).

d. Humoral Autoimmunity

Over the past decade, a number of investigators have reported that sera from patients with IDDM contain antibodies that react with pancreatic islet cells (Doniach and Bottazzo, 1977; Lernmark et al., 1978; Doberson et al, 1980a; Brogren and Lernmark, 1982). The spectrum of islet cell antibodies in IDDM encompasses several types of molecules with differing antigen specificities and other immunoreactive properties.

i. Islet Cell Cytoplasmic Antibodies (ICA)

In 1974, Bottazzo's group were the first to discover autoantibodies against the cytoplasm of islet cells in the sera of patients with longstanding IDDM. The method used to detect these antibodies involved cryostat sections of human pancreatic tissue from blood type O kidney donors to prevent interference from isoagglutinins. These

sections were incubated with sera from IDDM patients, and then indirectly stained with fluorescently labeled anti-human IgG. Initially, these islet cell antibodies (ICA) were thought to be present only in a rare subgroup of diabetic subjects with associated polyglandular autoimmune disorders. Subsequently, however, ICA were detected in the sera of about 60-70% (Irvine et al, 1977) of recently diagnosed insulin-dependent diabetics by a more sensitive technique. The low results obtained previously in patients with longstanding disease were explained by the subsequent demonstration that, in a large proportion of diabetic children, ICA tended to disappear from the circulation within the first few years after the onset of symptoms. Nevertheless the role of these ICA in the pathogenesis of IDDM remains dubious since ICA react with all the endocrine cell types within the islet including the glucagon-producing A cells, the somatostatin-producing D cells and the pancreatic polypeptide (pp) secreting cells, as well as the insulin secreting B-cells. ICA do not appear to be cytotoxic to islets, (Doberson et al., 1980) and moreover, they react with intracellular components. In general, living cells are impermeable to most antibodies. Therefore, the autoantigen may be inaccessible to the autoantibody in intact cells in vivo (Pujol-Borrell et al, 1982). In addition, similar cytoplasmic antibodies are also found in the sera of some patients with asymptomatic (Irvine et al., 1976) or latent diabetes (Del Prete et al., 1977).

These characteristics suggest that ICA may develop as a secondary response to islet cell damage. Nevertheless, ICA provide markers for potential IDDM patients and may prove valuable in the detection of the earliest stages of the disease process (Betterle et al., 1982). ICA

are present in approximately 9% of first-degree relatives of ICA positive probands compared to less than 0.5% of the population as a whole. The Barts-Windsor study in England evaluated the incidence of ICA in the first-degree relatives of patients with IDDM (Gorsuch et al., 1981). Of the 550 relatives screened, 54 had ICA. Seven of these patients developed IDDM within the three-year follow-up of the study. Patients without antibodies did not develop the disease. However, this does not rule out the possibility that ICA are produced as a consequence of beta cell destruction. It has been established that damage to beta cells begins long before the clinical manifestations of IDDM.

ii. Complement-fixing Islet Cell Antibodies (CF-ICA)

Bottazzo and coworkers (1980) have presented evidence for the existence of a separate class of islet cell antibodies which fix complement. The method for detecting these complement-fixing islet cell antibodies (CF-ICA) employs frozen sections of human pancreas and the antigen is cytoplasmic. However, the second antibody in this case is an anti-human-C3 or C1q conjugate applied to an immunofluorescent complement fixation test. Although it has been suggested that CF-ICA are more closely correlated with the onset of the clinical disease than ICA and that some CF-ICA selectively stain beta cells, there is still the question of how these antibodies could damage beta cells if they are solely directed against a cytoplasmic antigen. A direct cytotoxic effect of these antibodies on islet cells has not been demonstrated. Therefore, these antibodies could also be an epiphenomenon associated with the onset of IDDM, but not with the cause of the disease.

iii. Islet Cell-surface Antibodies (ICSA)

In contrast to the previous two autoantibodies, a different group of autoantibodies directed against the surface membrane of islet cells seem to possess more appropriate characteristics for involvement in the pathogenesis of IDDM. These antibodies bind specifically to beta cells but not to other islet cells (Van De Winkel et al., 1982), block insulin release in vitro (Kanatsuna et al., 1981), and exhibit cell-mediated (Maruyama et al., 1984a) and complement dependent cytotoxic properties (Soderstrum et al., 1979; Doberson et al., 1980a) which are consistent with a primary mediator of immune damage. MacLaren and coworkers (1975) reported the presence of islet cell surface antibodies (ICSA) in the sera from 87% of recent onset diabetic children using indirect immunofluorescence on a human insulinoma cell line. When tested on dispersed viable islet cells from rat or mouse, ICSA positive sera elicited a patchy cell-surface reaction which sometimes fused to form a continuous zone of fluorescence at the cell margin. The sera from more than 85% of recent onset diabetic patients who were ICSA positive, reacted only with the beta cell population. This was demonstrated by identification of cell types using electron microscopical analysis of ICSA-binding islet cells isolated by fluorescence-activated cell sorting. The immunocytochemical characterization of these ICSA-positive cells was accomplished using antibodies to insulin, glucagon, somatostatin or pancreatic polypeptide. Cells which bound ICSA were found to counter-stain with anti-insulin antibody only. In addition, these antibodies, like ICA, are prevalent at the time of diagnosis but decline thereafter and are not species specific.

Several studies with guinea pig serum as a source of complement indicate that islet cell autoantibodies in human serum can mediate cytotoxicity as measured by an increase in chromium-51 release from radiolabelled rat islet cells, rat insulinoma cells or dispersed human islet cells (Doberson et al., 1980; Eisenbarth et al., 1981). Cytotoxic islet cell antibodies correlated with ICSA but not cytoplasmic antibodies (Doberson et al., 1980). In another experiment, the uptake of ethidium bromide was used to identify lysed cells in rat islet cultures which had been exposed to IDDM sera containing ICSA (Doberson and Scharff, 1982). Immunofluorescent staining with antisera to insulin, glucagon, somatostatin, or pancreatic polypeptide was used to identify the different cell types. The results showed that in the presence of complement, sera containing ICSA lysed 81% of the beta cells but less than 10% of the A-, D- and pp-cells, whereas normal control sera lysed less than 4% of each of the cell types. Furthermore, insulin-secreting column-perfused rat beta cells, following exposure to rabbit anti-serum against rat islet surface antigens together with complement, were unable to release insulin in response to glucose stimulation following an initial transient release (Kanatsuna et al., 1981). In addition, islet cell function was shown to be debilitated when 50% of IDDM sera were found to suppress the insulin release from isolated mouse islet cells stimulated by glucose and theophylline (Sai et al., 1981). Morphological evidence and the trypan blue dye exclusion test have suggested that the suppression of insulin release was due to a cytotoxic effect of the sera.

Maruyama et al. (1984a) and Charles et al. (1983) both demonstrated ICSA-dependent cell-mediated cytotoxicity to pancreatic beta cells

in the presence of non-idiotypic lymphocytes. Four out of 14 ICSA positive complement-depleted sera from patients with IDDM showed a cytotoxic reaction to cloned human fetal pancreatic beta cells by a 51-chromium release assay in the presence of normal human lymphocytes (Maruyama et al., 1984a).

These observations suggest a possible etiological involvement of cytotoxic ICSA in the pathogenesis of IDDM via a complement-fixation route as well as by antibody dependent cell-mediated cytotoxicity (ADCC). There is little doubt that the presence of ICSA in IDDM is an interesting occurrence and certainly worthy of further study, however the exact role of ICSA in the pathogenesis of IDDM remains to be elucidated.

Some of the evidence discredits the relevance of these antibodies in the pathogenic process. For example, the frequency of ICSA in patients with IDDM varies considerably between studies (32% - 94%) (Lernmark et al., 1978; Van De Winkel et al., 1982), and these antibodies are also found in a significant proportion of first degree relatives (Doberson et al., 1980a). Using a ^{51}Cr-release assay Dobersen and coworkers demonstrated that 25% (14/56) of nondiabetic first degree relatives of diabetic probands exhibit cytotoxic ICSA. In another study, sera from 21% of nondiabetic, age-matched pediatric patients were ICSA positive by indirect immunofluorescence (Grant et al., 1985). In addition, no conclusive data are available to determine whether there is a correlation between the presence of cytotoxic ICSA and the onset of diabetes. Therefore, it is not yet clear if these autoantibodies play a primary role in the destruction of beta cells or if they

are a secondary phenomenon associated with the onset of IDDM but not causing the disease.

One way to verify whether ICSA are primary mediators would be by transfer experiments. However, few attempts to induce insulin-dependent diabetes in animals by the transfer of serum from type 1 diabetics, have been documented. When IgG from 6 insulin-dependent diabetic patients was transferred into mice by daily intraperitoneal injections for 7 days none of the animals became diabetic. However all the mice showed an impaired glucose tolerance by the 7th day (Assan, reported in Kolb and Kolb-Bachofen, 1983). The interpretation of these results is difficult as Maclaren has also observed elevated glucose levels in mice which received serum from patients with Myasthenia gravis (reported in Kolb and Kolb-Bachofen, 1983).

Little progress has been made, as yet, in characterizing and purifying the target autoantigen using sera from IDDM patients containing ICSA. Preliminary studies have identified a 64-kD protein as the beta cell specific target antigen of ICSA from detergent lysates of human islet cells (Baekkeskov et al., 1982). ICSA directed against this antigen were observed two years before the onset of diabetes in the discordant twin of a diabetic who did not have detectable islet cell cytoplasmic antibodies. These workers suggest that the 64-kd determinant represents a major target antigen involved in the pathogenesis of IDDM. However, the biochemical composition of this antigen has yet to be described.

B. Heterogeneity in IDDM

There appears to be a great deal of heterogeneity within the subgroup of diseases known as IDDM. Inconsistencies in the presence or absence of many of the autoimmune phenomena exist between diabetic individuals and many of the immune abnormalities are present in first degree relatives who are unaffected by IDDM. Genetic heterogeneity also exists and it is therefore likely that there is more than one cause for the destruction of beta cells apparent in IDDM.

As discussed previously, in a small percentage of IDDM cases viruses may be directly responsible for the destruction of beta cells without any apparent input from autoimmune reactions (Yoon et al., 1979). Chemical toxins have also been implicated in a few cases.

For the majority of insulin-dependent diabetics who show overt symptoms early in their life, the etiology is most likely to involve environmental factors such as viruses which trigger an autoimmune response. Possibly, these individuals have a genetic susceptiblity to a pancreatropic viral infection which initiates islet cell autoimmunity, thus triggering the production of autoantibodies and autoreactive T cells.

Although in most cases an environmental agent is suspected to initiate the chain of events leading to the development of autoimmunity, in a small subset of IDDM patients, autoimmune reactions appear to arise spontaneously. These patients are characterized by concomitant autoimmunity against several other endocrine glands including thyroid, adrenal and in some instances gastric parietal cells (Irvine et al., 1970; Bottazzo et al., 1974). IDDM under these circumstances may be one manifestation of a broad range of anti-self-reactivity.

Approximately 10% of IDDM patients belong to this subgroup which are mostly represented by older females. These patients, in turn, belong to a larger group of individuals with an autoimmune syndrome termed Type II polyendocrine disease which also includes idiopathic Addison's disease, autoimmune thyroid disease, myasthenia gravis and pernicious anemia (Bottazzo et al., 1984). It appears that in polyendocrine diabetes, or type Ib as referred to by Bottazzo and coworkers (1984), there is a slower destruction of the pancreatic islets with residual C-peptide secretion (a reliable indication of endogenous beta cell secretory reserve) persisting for some years. This minority of IDDM patients exhibit clinical and serological features often described in polyendocrine autoimmunity. For instance, ICA persist for many years in these individuals as compared to the transient appearance in the more common form of diabetes (Bottazzo et al., 1984). Patients with type II polyendocrine autoimmune syndrome are likely to have a direct autoimmune etiology which is linked to genetic susceptibility. This is associated with histocompatability antigens HLA-B8 and -DRW3 and there appears to be a link between these antigens and islet cell antibody positivity in this subgroup of patients although this is controversial (Doniach et al., 1982). In some ways the spontaneous occurrence of IDDM in this syndrome is reminiscent of the type of diabetes observed in the BB rat animal model which will be described in the following section.

In summary, a few cases of IDDM may be caused by a direct, severe, virus-induced injury of the beta cells; in other infrequent cases, it may present as a component of polyendocrine autoimmunity. In the great majority of cases, however, IDDM results from a complex interplay of

environmental (possibly viral), genetic (HLA-linked) and immunological factors.

C. Animal Models for IDDM

As discussed earlier, the inheritance, etiology, physiology, therapy and prevention of human IDDM are still unresolved problems. Therefore, the use of appropriate animal models of IDDM are necessary and indeed have provided valuable insights into the disease. Animal models provide the means to study biopsy and autopsy materials. It is possible to provoke the disease in animals, as well as testing new methods of therapy. Furthermore, selective breeding can be carried out in animal models. However, it is unlikely that any animal model will be absolutely identical to human diabetes and choice of the most appropriate model is crucial.

Although many models of spontaneously diabetic laboratory animals have been studied in the past 25 to 30 years, few are true examples of non-obese, insulin-dependent, ketosis-prone diabetes. Almost all of the spontaneously diabetic animal models evidence obesity, beta cell hyperplasia, and increased pancreatic and circulating insulin (Like, 1977), which are not characteristic features of human type 1 diabetes.

Two exceptions exist which have many of the same characteristics as human IDDM. Recently, the 'non-obese-diabetes' or NOD mouse was reported from Japan (Makino et al., 1980). NOD mice have abnormalities which are characteristic of autoimmune diseases, such as fewer T-lymphocytes and exaggerated antibody production against exogenous stimuli (Maruyama et al., 1984b). The appearance of insulitis and islet cell cytoplasmic and surface antibodies as well as many other

cellular and humoral abnormalities have been observed before the onset of ketosis-prone diabetes in the NOD mice. Beginning at 4-5 weeks of age, the lymphocytic infiltration is progressively and selectively associated with pancreatic beta cell destruction. Diabetes occurs between 13 and 30 weeks, with hyperglycemia and ketonuria, and in some instances the complete requirement of insulin. Testosterone levels seem to influence the appearance of diabetes in NOD mice, the disease being more frequent in females (80-90% by 30 weeks) than in males (20%). However, lymphocytic infiltration occurs by 5 weeks in both sexes (females 82%, males 58%) (Fujita et al., 1982). Although the NOD mouse appears to exhibit a syndrome similar to human IDDM more work is required to further characterize this model.

1. The BB RAT

a. "Background"

The Biobreeding or BB rat is probably the most important animal 'model' for Type 1 diabetes and one which has already greatly contributed to a better understanding of possible pathogenic mechanisms of autoimmunity in islet cell damage. A review of the BB rat is provided by Marliss et al. (1982). First recognized in 1977, the BB rat is a diabetic strain of Wistar-derived rat accidentally discovered as a spontaneous mutation in a commercial breeding colony in Ottawa, Canada (Chappel and Chappel, 1983).

The original BB colony was established by cross breeding the normal parents of diabetic animals. Initially, only 10% of the offspring of these matings became diabetic, but with selective breeding

the frequency of diabetes has been substantially increased. All BB rats are descended from the original Ottawa litters, but rats in different colonies vary considerably in the frequency and severity of diabetes.

b. Natural History

Approximately 50% of the rats develop insulin-dependent diabetes although incidences ranging from 30-70% have been reported. Onset of diabetes is abrupt. Most diabetic animals succumb from ketoacidosis within one week of onset unless insulin is given. The age at which diabetes develops is relatively consistent, generally between 60 and 120 days of age in most colonies. The mean age of detection of diabetes in more than 1,000 diabetic rats studied in the colony at the University of Massachusetts Medical Center was 92.4 days with only 15-20% of animals becoming diabetic after 120 days of age (Like and Rossini, 1984). The onset of diabetes, therefore, coincides with the age of sexual maturity. Variable but usually small numbers of animals become glucose intolerant and do not progress to overt diabetes. Once the animals are persistently glycosuric they become permanently diabetic. Insulin-dependent rats that spontaneously enter remission are very rare (Marliss et al., 1983).

c. Physiological and Biochemical Features

Polyuria, polydipsia, hyperglycemia and glycosuria become manifest abruptly in animals which, in general, could not be distinguished from normal littermates only a few days previously. Markedly depressed plasma insulin levels and elevated glucagon levels have been reported

(Nakhooda et al., 1976). Ketone bodies and circulating free fatty acid concentrations are also elevated in animals that do not receive exogenous insulin therapy. Plasma glucose levels in untreated diabetic BB rats are greater than 250 mg/dl and are often greater than 500 mg/dl (Nakhooda et al., 1978). In addition, hyperaminoacidemia and a generalized catabolic state are suggested by elevated 3-methylhistidine and branched chain amino acid concentrations and by increased ammoniagenesis.

d. Morphological Findings: Insulitis

Inflammatory lesions of the pancreatic islets which resembles that of IDDM in man often precede the onset of diabetes in BB rats (Nakhooda et al., 1978). This pancreatic infiltrate has been shown to consist predominantly of cells which react with OX4 monoclonal antibody, indicating that they are Ia-positive cells. This implies that they are activated T lymphocytes and/or macrophages (Like et al., 1983). Recently, it was reported that the major population of infiltrated cells appeared to be macrophages identified by the OX-41 monoclonal antibody (Kim et al., 1987). Other cells present in decreasing numbers, include helper/inducer cells indicated by W3/25 monoclonal antibody and cytotoxic/suppressor cells indicated by OX-8 monoclonal antibody. Islets from rats sacrificed at the time of onset of diabetes contain virtually no beta cells. The number of A, D and pp cells are also decreased. Extraction of islet hormones from diabetic rats confirms the absence of insulin and the decrease of other islet hormones (Patel et al., 1983). Insulitis is generally absent in long term, insulin-treated diabetic rats.

e. Immunological Abnormalities

A universally reported phenotypic feature of diabetic-prone BB rats is the presence of T cell lymphopenia. A number of studies have demonstrated that diabetic and prediabetic BB rats are profoundly lymphopenic, in the helper/inducer and cytotoxic/suppressor subset of T lymphocytes (Jackson et al., 1981; Like et al, 1983). It has been suggested that lymphopenia is essential for the development of diabetes in this animal. Concomitant with the lymphopenia, BB rats are susceptible to environmental pathogens (Like et al., 1982a) and malignant lymphomas (Seemayer et al., 1983). Autopsy studies of diabetic BB rats revealed 30% of the animals to have significant pneumonia (Seemayer et al., 1983). BB rats have also been shown to be particularly vulnerable to mycoplasma.

f. Evidence Supporting an Autoimmune Pathogenesis in the BB Rat

The observation of insulitis was only the first of many lines of evidence to support the hypothesis of an autoimmune pathogenesis of diabetes in the BB rat. Other important supportive data have come from studies of humoral and cell-mediated immunity which are described in the following sections.

i. Humoral Immunity

The possibility that immunopathological mechanisms are involved in the development of spontaneous diabetes was supported by the presence of circulating autoantibodies in newly diagnosed BB rats (Marliss et al., 1982; Elder and Maclaren, 1982a). There is no evidence for the presence of islet cell cytoplasmic antibodies in these animals (Like et

al., 1982b). On the other hand, antibodies to the surface of islet cells have been reported by many workers (Pollard et al., 1983; Dyrberg et al., 1982; Martin and Logothetopoulos, 1984). A great deal of variation in the levels of ICSA exists between different studies and indeed within and between individual rats. Whether a direct correlation exists between the presence of ICSA and diabetes remains controversial.

In an indirect immunofluorescent assay on rat islet cell suspensions, Pollard and coworkers (1983) found 12 out of 12 diabetic rats had ICSA. This would appear to be a close correlation, but 4 out of 12 prediabetic BB rats consistently showed ICSA and did not develop diabetes. In an ^{125}I-protein A radioligand assay only 32% (7/22) of the high-incidence BB rats exhibited ICSA when the mean binding values during 38-157 days of age were compared (Dyrberg et al., 1982). Five out of the 22 rats developed diabetes without expressing ICSA and 4 were ICSA positive but did not become diabetic. In this study Dryberg and coworkers concluded that there was no correlation between the development of diabetes, glucose intolerance or insulitis and the level of ICSA. They found that 50% of the diabetic rats were ICSA positive at only 50 days of age. However, some of the rats showed peak ICSA levels at or after the onset of diabetes, therefore, ICSA may not be present before the diagnosis of diabetes in all rats. In a very recent report 41% of high incidence BB rats (n = 48) developed diabetes and ICSA whereas 17% became diabetic without exhibiting detectable levels of ICSA (Dean et al., 1987). An additional 10% were ICSA positive but remained healthy at 150 days.

In contrast, in a cytolytic assay using islet cells prelabeled with ^{3}H-leucine, Martin and Logothetopolous (1984) found that sera from 13 out of 14 diabetic BB rats showed complement-dependent cytotoxicity against islet cells, and in the majority, the cytotoxic ICSA appeared before the onset of diabetes. Nevertheless, 6 of the rats developed positive sera no earlier than 14 days prior to the diagnosis of diabetes. The number of rats exhibiting ICSA but not diabetes was not mentioned in this paper. Although ICSA are certainly an interesting phenomenon in insulin-dependent diabetes their relevance to the pathogenesis of the disease remains unclear, and indeed this area requires further study.

As mentioned previously, Baekkeskov et al. (1982) showed that human ICSA positive sera immunoprecipitated a 64-kD protein from normal human islets of Langerhans isolated from cadaveric kidney donors. In an attempt to identify the antigen(s) detected by BB rat ICSA, homogenates of normal rat islets were immunoprecipitated with BB rat serum (Baekkeskov et al., 1984). As a result, sera from 25 out of 26 diabetes-susceptible BB/Hagedorn rats reacted with a 64-kD rat islet cell protein which was not present in rat spleen lymphocytes. A prospective analysis in 6 of these rats showed the presence of ICSA, 40 to 70 days, before diabetes onset in 5 of the rats. In the other animal, antibodies to the 64-kD protein were detected only in a serum sample obtained at the onset of IDDM and 28 days later. In the same report, 6 out of the 16 control low incidence BB rats sera also precipitated this protein. Nevertheless, it remains to be established if the human and the rat 64-kD islet cell surface proteins are identical.

Other autoantibodies can be found in BB rats. Anti-lymphocyte antibodies are also prevalent (Dyrberg et al., 1984b) as are autoantibodies which bind to smooth muscle, thyroid colloid and gastric parietal cells (Like et al., 1982b).

Attempts have been made to transfer diabetes via diabetic sera to assess the importance of humoral autoimmunity in the pathogenesis of IDDM. Injection of serum from diabetic rats has not produced insulitis or diabetes in BB recipients. However, further transfer experiments are required before a final conclusion on the relevance of autoantibodies in BB rats can be reached.

ii. Monoclonal ICSA

The production of monoclonal ICSA, using hybridoma technology, would provide a useful tool for analyzing the role of these autoantibodies in IDDM. A monoclonal autoantibody (IC_2), produced by the fusion of spleen cells from a BB rat and a rat myeloma cell line has been produced by Brogren and coworkers (1986). Although this monoclonal antibody was reported to bind to a rat insulinoma cell line (RINm5F) in a cellular enzyme linked immunosorbent assay and to normal rat islet cells by immunofluorescence, IC2 did not lyse RINm5F cells or rat islet cells, nor was a target antigen identified. A cytotoxic rat monoclonal autoantibody (E5C2) that reacts with RINm5F cells and primary rat islet cells has recently been reported (Uchigata et al., 1987). Neuraminidase treatment of rat islet cells was necessary before the cells were susceptible to complement-mediated lysis by antibody E5C2. The workers suggest that exposure of hidden antigenic determinants may trigger and be the target of autoimmune attack. Yet the ICSA

in the sera of human diabetics, BB rats and NOD mice recognize antigens on normal untreated islet cells and it has been suggested by a number of workers that the underlying defect in IDDM is more likely to be an abnormality in immune regulation rather than an antigenically altered target cell recognized by a normal immune surveillance system (Like et al., 1982b; Prud'homme et al., 1984). To date, there have been no reports of a rat monoclonal autoantibody which is cytotoxic to both untreated RINm5F and cultured islet cells. Such a monoclonal ICSA would be a close representation of the typical autoantibodies found in vivo and may prove a useful tool in analysing the role of ICSA in the pathogenesis of IDDM.

iii. Cell-Mediated Immunity

In contrast to the argument for a humoral etiology, convincing evidence for a cell-mediated immune process in the pathogenesis of insulin-dependent diabetes comes from transfer experiments in BB rats. Peripheral and splenic lymphocytes from newly diagnosed diabetic BB rats were first reported to transfer insulitis passively when given to athymic mice (Nakhooda et al., 1981). Repeated injections of the cells increased the frequency and intensity of the insulitis. However, no hyperglycemia could be detected in the recipients, even when stressed with a glucose tolerance test (Neufeld et al., 1979). However, adoptive transfer of both insulitis and diabetes was accomplished when spleen cells from acutely diabetic BB rats were cultured in the presence of concanavalin A prior to transfer into prediabetic BB rat recipients (Koevary et al., 1983a). Diabetes occurred in a large percentage of the young BB rats well before the expected spontaneous

onset of hyperglycemia. Moreover, using the same protocol, they also adoptively transferred diabetes to diabetes resistant Wistar-Furth rats (Koevary et al., 1983b).

A salient feature of the BB rat lies in the success of immunotherapy in preventing and even curing the disease. Injection of antiserum against rat lymphocytes into BB rats prevents and ameliorates the diabetic syndrome (Like et al., 1979). Neonatal thymectomy (Like et al., 1982c) or bone marrow transfusions (Naji et al., 1981) are found to prevent or reduce the frequency of diabetes, respectively. The most exciting recent finding, which is now being applied to human diabetics, was the discovery that treatment with cyclosporin A prior to the onset of overt diabetes can prevent the disease in BB rats (Laupacis et al, 1983). Cyclosporin A is believed to act by inhibiting T-lymphocyte proliferation in response to T cell-dependent antigens. It is thought to affect principally the helper/inducer T lymphocyte by arresting its maturation and thus inhibiting interleukin release while sparing suppressor T lymphocytes. This prevents the generation of cytotoxic lymphocytes, therefore ameliorating the T cell-mediated immune response. In contrast, recent reports have indicated that cyclosporin A can induce hyperglycemia and hypoinsulinemia in low-incidence BB rats (Eun, 1987). The mechanism of action of the adverse effects of cyclosporin A is not known, therefore it is clear that caution must be exercised if cyclosporin A were to be used in high risk, clinically nondiabetic, first degree relatives of patients with IDDM.

g. Genetics of the BB Rat

The diabetic syndrome in the BB rat is hereditary with strong

evidence suggesting an autosomal recessive mode of inheritance with incomplete (about 50%) penetrance (Butler et al., 1983), possibly involving three separate genes (Guttman et al., 1983), certainly two (Buse et al., 1984). One gene is thought to determine the T-cell lymphopenia, the second appears to be associated with RT1, the major histocompatability complex of the rat. A third component, pancreatic lymphocytic infiltration, has also been implicated as a separate factor required for the full expression of the diabetic state (Guttman et al., 1983). A recent study has employed restriction endonucleases to determine RT1 class II-gene polymorphisms in the BB rat (Buse et al., 1984). Using a mouse MHC cDNA probe, four polymorphic chromosome types were found in diabetes-resistant BB rats from the Ottawa colony, only one of which is found among diabetes prone BB rats. Studies such as this should aid in the localization and eventual characterization of the RT1 linked diabetogenic gene of the BB rat.

D. Concluding Comments on Possible Causes of IDDM

Although much data exists for both cellular and humoral autoimmune processes directed at the pancreatic islet cells in IDDM, the evidence for autoimmune mechanisms playing a role in the pathogenesis of IDDM is mainly indirect. There are many inconsistencies in the results from the different research groups making interpretation of autoimmune involvement difficult. However several theories have been proposed.

The demonstration of ICSA-dependent cell-mediated cytotoxicity to pancreatic B cells in vitro, taken together with the reported increase in K cells and the presence of immune complexes, suggesting excess antibody, makes it tempting to speculate that antibody-dependent

cell-mediated cytotoxicity (ADCC) may be important in the etiology of IDDM. However, the evidence is purely circumstantial and increases in the level of both natural cytotoxicity and ADCC may be a consequence of disturbed metabolism rather than a cause of it. It is interesting that a decrease in the number and activity of suppressor T cells and an increase in the level of Ia-positive T cells has been reported in IDDM as well as a number of other autoimmune disorders. These events may bring about the proliferation of autoreactive T- and B-cells. This is possibly genetically linked on account of the association of certain HLA haplotypes with autoimmune phenomena in IDDM.

A hypothesis for the induction of autoimmunity in genetically susceptible individuals has been presented by Adams and coworkers (1984), which is based on the forbidden clone theory of Burnet (1959). This theory relies on the proposition that during ontogeny, clonal deletion of B lymphocytes which recognize the individuals own histocompatibility antigens takes place. Such deletions would influence the chance appearance of autoreactive forbidden clones by somatic mutation of 'pre-forbidden' clones. The effect could be to diminish or enhance the risk of autoimmunity depending on which histocompatibility antigen alleles the individual possesses. For example, as already mentioned HLA-DR2 confers protection from diabetes. The theory argues that protection arises because clones which recognized HLA-DR2 and were deleted, were the clones that, after somatic mutation of their V regions, could have reacted with islet cell autoantigens. By contrast HLA-B8 predisposes to diabetes. In this case, the B lymphocyte clones directed against HLA-B8 may have the potential to produce anti-idiotypic antibodies to idiotypes which after appropriate somatic

mutation may proceed to recognise islet-cells. The deletion of these anti-idiotype clones might release from suppression these "pre-forbidden" idiotypes. This is an interesting theory but it requires supporting evidence.

A possible hypothesis as to how viruses may trigger the autoimmune destruction of beta cells is derived from the observation that HLA-DR antigens have been found on the surface of islet cells from patients with IDDM (Baekkeskov et al., 1981). As mentioned previously, HLA-D antigens are expressed on antigen-presenting cells of the immune system and are not normally expressed on resting endocrine cells under physiological conditions. Recently it has been demonstrated, however, that normal thyroid cells in culture can be stimulated by mitogens to express HLA-DR molecules on their plasma membranes. Moreover, thyrocytes isolated from patients with Graves' disease do not require any mitogenic stimulus and were found to spontaneously show DR molecules when cultured in monolayers. Preliminary experiments with human diabetic pancreas have shown that some of the cells in diseased islets stain with immunofluorescently labelled monoclonal anti-DR antibodies (Bottazzo et al., 1983a). The shape and size of the DR-positive cells suggest that they may be endocrine rather than immune cells. Bottazzo (1983b) has speculated that viruses, which might be present in the pancreas of genetically predisposed individuals without causing any signs of infection, may stimulate the production of interferon. Interferon in turn might induce the expression of HLA-DR antigens on beta cells which then act as antigen presenting cells for self antigens and subsequently induce autoreactive T helper cells. T helper cells generally only recognize antigens that are associated with class II molecules. Therefore, the interaction of the T-cell receptor, the

autoantigen and the class II molecule could lead to T cell activation. These T-cells would in turn activate effector B- and T-lymphocytes with the resulting production of ICSA and cytotoxic T cells against beta cells. Further studies are required to determine whether it is beta cells which are inappropriately expressing DR antigens in diabetic insulitis. However even if, for example it is dendritic cells within the pancreas, which are expressing HLA-DR antigens a bystander effect could produce the same results. Perhaps the transition from the initial induction of T cells to the fully developed autoimmune disease depends on a variety of other factors such as deficiencies in the suppressor T-cell pathway.

Although the etiology of IDDM is far from being understood, it is clear that autoimmunity is involved. Whether cell-mediated or humoral autoimmunity (or both) are primary mediators of beta cell damage is open to question. It is not yet clear if cytotoxic ICSA play a primary role in the destruction of beta cells or if they are a secondary phenomenon associated with the onset of IDDM but not causing the disease. Therefore, further work is required to determine whether there is a close correlation between cytotoxic ICSA and diabetes. Investigation of these autoantibodies is necessary and their in vivo function needs to be elucidated. In the event that IDDM has a primarily cell-mediated pathogenesis, the key to identification and isolation of the autoantigen may lie with the ICSA. These antibodies may also provide the tool, possibly in the form of antiidiotypic antibodies, which could supply access to the T cells that may be targeting the same antigen on the beta cells.

II. GENERAL OBJECTIVES

It is apparent from the previous review of the literature that there is extensive evidence which suggests that in a large number of patients, IDDM may result from an autoimmune process directed at the insulin producing pancreatic beta cells. Antibodies which react with the islets of Langerhans have been demonstrated in patients with IDDM by many different workers. Among these antibodies, complement-mediated cytotoxic antibodies specific for a beta cell membrane antigen(s) appear to be most closely correlated with the pathogenesis of the disease, but direct evidence for their causative role is lacking. It is, therefore, important to determine the extent to which the appearance of islet cell surface antibodies indicates islet cell destruction. One way to evaluate the possible role of ICSA in the pathogenesis of IDDM is to ascertain whether there is a close correlation between the presence of ICSA and overt diabetes in BB rats. Furthermore, it would be useful to determine if ICSA develop prior to the clinical onset of diabetes. Therefore, the first objective of this research project was to examine the association between the presence of cytotoxic ICSA and the development of overt diabetes in our BB rat colony. The second objective was to produce a monoclonal cytotoxic autoantibody against an islet cell surface antigen using standard hybridoma techniques. A monoclonal autoantibody of this nature would clearly be a useful tool for analyzing the importance of these autoantibodies in diabetes and for investigating the autoantigen(s) to which they are directed.

III. SPECIFIC OBJECTIVES

A. ICSA in vivo

Answers to the following questions were sought.

1. Is there a correlation between diabetes and cytotoxic ICSA in our BB rat colony?
2. Do cytotoxic ICSA precede overt diabetes?
3. Are there differences in the incidences of cytotoxic ICSA and overt diabetes between BB rats from different sources?
4. Are there gender-related differences in the presence of cytotoxic ICSA?
5. Is it possible to induce overt diabetes by induction of cytotoxic ICSA?

B. ICSA in vitro

1. Production of a monoclonal cytotoxic ICSA.
2. Purification of the monoclonal ICSA.
3. Characterization of the monoclonal ICSA (i.e. analysis of specificity, cytotoxicity and binding properties).
4. Characterization of autoantigen to which the monoclonal ICSA is directed (i.e. determination of molecular weight of autoantigen).

MATERIALS AND METHODS

A. Animals

The high-incidence BB rats used in this study were from two sources. Thirty-six male rats came from the University of Massachusetts colony and will be referred to as BB/W rats. The other 29 rats (12 males and 17 females) were the progeny of rats obtained from Dr. P. Thibert (Animal Resources Division, Health Protection Branch, Ottawa, Canada) and will be referred to as BB_o rats. The two colonies were not allowed to interbreed and the BB_o rats in the study were from the 2nd or 3rd generations bred at our facilities. The onset of diabetes was determined by directly testing the urine biweekly with Tes-Tape (Eli-Lily Canada Inc., Toronto, Ontario). Individual rats were classified as diabetic on the basis of a positive glycosuria and they were subsequently maintained on daily PZ1 insulin. The prevalence of diabetes was 43% among the 36 BB/W rats and 65% among the 29 BB_o rats. To determine the presence of cytotoxic ICSA, blood samples were obtained from the retro-orbital sinus under light ether anaesthesia. Blood samples were collected approximately every 7 days starting, in some cases, as early as 40 days of age and continuing up to about 150-160 days of age. The blood was allowed to clot then centrifuged at 2000 xg for 20 minutes. The sera was used either the same day or the next day in a ^{51}Cr-release cytotoxic assay as will be described later.

B. Rat Insulinoma Cells

The rat insulinoma cell line (RINm5F) as established by Gazdar et

al. (1980) was obtained from the National Institute of Health, Bethesda, Maryland, USA. RINm5F is a continuously growing, insulin producing islet cell line cloned from cells originating from a transplantable radiation-induced rat islet cell tumor (Gazdar et al., 1980). Cloned beta cells are comparitively homogeneous and in that respect they are better than primary cultures of mixed endocrine cells from the islets of Langerhans. This eliminates the variability from batch to batch which occurs when using primary islet cell cultures. In addition, they allow screening of a larger number of serum or hybridoma supernatant samples while avoiding the time-consuming process of isolating islet cells. It has previously been shown that the reactivity of BB rat sera to RINm5F cells correlates with that observed with dispersed normal Wistar rat islet cells (Dyrberg, et al., 1982). Therefore, RINm5F were used as target cells in this study when screening for cytotoxic ICSA. Monolayers of the RINm5F cells were incubated at 37°C in RPMI 1640 culture medium (Gibco) supplemented with 10% fetal calf serum (FCS) in an atmosphere of 5% CO_2.

C. Islet Cell Cultures

Cultures of neonatal Wistar Rat pancreatic islets were prepared according to the method of Yoon et al. (1984) which is an adaptation of the method of Dobersen and coworkers (1980b). Briefly, the pancreases of approximately 30 neonatal Wistar rats (10-20 days old) were removed and the tail portion was dissected and washed in 5 changes of cold (4°C) calcium-magnesium PBS. The pancreatic tissue was then minced, washed again in PBS and transferred to prewarmed collagenase solution (Cooper Biomedical, specific activity 145 u/mg; 12 mg/ml in PBS).

Enzymatic digestion was carried out for 20-25 minutes at 37°C with magnetic stirring. Large clumps of exocrine tissue were removed by swirling a sterile 9 inch Pasteur pipette through the digestant. Three ml aliquots of the collagenase treated material were transferred into 15 ml conical tubes containing 10 ml of cold PBS. The tubes were centrifuged at low speed (1-2 minutes at 1000 xg) and the supernatant was discarded. The cell suspension was washed one more time and then filtered through two layers of sterile gauze to remove any aggregates. The filtrate was then centrifuged at 2000xg for 7 minutes at 5°C. The pellet was resuspended in prewarmed (37°C) growth medium and seeded into 35 ml culture dishes. The cell concentration used for seeding would depend on the yield of islets obtained. Following 24 hours the islet cells would be decanted into new 35 mm dishes and most of the fibroblastoid cells would remain attached to the original culture dishes.

D. Myeloma Cells

The mouse myeloma cell line SP2/0 was used in the fusion which produced the monoclonal antibody KT1. Other myeloma cell lines used were P_3 X 63Ag653 and the rat myeloma cell lines Y3-Ag1.2.3 and YB2/0. However monoclonal antibodies with the required specificity were not obtained from fusion involving these myeloma cells.

E. Control Cell Lines

IEC-18 (rat ileum epithelial cell line), L-2, Vero, BHK and MKD cells were used to check for cross-reactivity of the monoclonal antibody. IEC-18 was obtained from the American Type Culture Collection

and the other lines were kindly donated by Dr. R. Anderson and Dr. P. Lee, Department of Microbiology and Infectious Diseases, University of Calgary.

F. Isolation of Lymphocytes

Rat lymphocytes used in the production of hybridomas were obtained from the spleens of prediabetic BB rats which had a high incidence of spontaneous insulin-dependent diabetes. Sera from the BB rats were first screened by a ^{51}Cr-release assay to measure cytotoxic ICSA. Rats which were high responders were used in the cell fusions.

G. Precursor Enrichment

A simple procedure for pre-selecting lymphocytes of the desired specificity was adapted from the method described by Winger and coworkers (1983). Briefly, insulinoma cells were allowed to adhere to a 90 mm diameter tissue culture Petri dish (Corning). Isolated lymphocytes in DMEM containing 10% fetal calf serum were allowed to settle in the dish for 1 hour at $4^{o}C$. Nonadherent cells were removed by gentle swirling. Adherent cells were removed by vigorous pipetting and washed twice with DMEM prior to fusion.

H. Production of Hybridomas

Hybridomas were produced according to the techniques initially developed by Kohler and Milstein (1975), but since an autoimmune response was under investigation the BB rats were not immunized. The BB rats were bled from the retro-orbital sinus and their sera were screened for the presence of cytotoxic islet cell surface antibodies by

the microcytotoxicity assay described in the following section. Some of the rats which exhibited ICSA were boosted with RINm5F cells, either by intraperitoneal or tail-vein injection, 5 to 7 days prior to fusion. Single cell suspensions of spleen cells from animals exhibiting high titers of ICSA were fused with the SP2/0 mouse myeloma cell line. Cells were counted by trypan blue exclusion and mixed at a ratio of 5 spleen cells per myeloma cell. (When rat myeloma cells were used in the fusion the ratio was 2 spleen cells to 1 myeloma cell). This mixture was co-pelleted by centrifugation at 400xg for 10 minutes and fusion was accomplished by adding 1.0 ml of 50% polyethyleneglycol 4000 (PEG; Serva, New York) in DMEM at 37^{o}C over 1 minute while stirring with a pipette tip. After stirring for a further minute, the cell suspension was sequentially diluted by slowly adding 20 mls DMEM without fetal calf serum over a time of 2-5 minutes, dropwise at first, and then in gradually increasing volumes. This was allowed to stand for 2 minutes, then centrifuged and resuspended in HAT medium containing 20% fetal calf serum. HAT medium which is a selective medium consisting of 4 X 10^{-9} M aminopterin, 7 X 10^{-5} M adenine, 1.6 X 10^{-5} M thymidine in DMEM, allows for the selection of fusion products according to the method of Littlefield (1964), while FCS arrests the action of PEG. In a modified method, the cells were incubated for 1 hour at 37^{o}C without disturbing the pellet, prior to sequential dilution in DMEM. This was found to produce a higher fusion efficiency and was adopted from then on. 0.1 ml aliquots of the cell suspension, containing 5 X 10^{6} cells/ml, were added to each well of a 96 well tissue culture plate which was previously seeded with a feeder cell suspension

of thymocytes in 0.1 ml HAT medium. The plates were incubated at 37°C in a humidified incubator containing a 10% CO_2 and 93% air atmosphere.

I. Microcytotoxic Assay Using RINm5F Cells as a Substrate

Monolayers of RINm5F cells, in 96 well microtiter plates, were incubated in medium containing [^{51}Cr] sodium chromate (Amersham, 100-400 mCi/mg chromium, 1 uCi/well) for 5 hours at 37°C. They were then washed and exposed to 100 ul of a 1:2 dilution of rat sera or undiluted hybridoma supernatant for 30 minutes at 37°C. Following further washing, the monolayers were incubated with 200 ul of a 1:5 dilution of low-toxicity rabbit complement (Cedar Lane Laboratories) for 40 minutes at 37°C. Aliquots of each reaction mixture were counted on a gamma counter and the toxicity (lysis of beta cells) was determined by the following equation: % lysis = 100 x [(^{51}Cr release in the presence of supernatant + complement) - (^{51}Cr release with complement alone)/[(total ^{51}Cr release by Triton X-100 treated cells) - (^{51}Cr release with complement alone)]. Results given are the averages of duplicate determinations. A hyperimmune serum prepared in rabbits against RINm5F cells served as a positive control. In the initial assays for screening rat sera, preabsorption with rat liver powder was carried out but this was not found to change the level of cytotoxicity when compared to unabsorbed sera. It was therefore, decided to eliminate this unnecessary step.

J. Microcytotoxic Assay of Monoclonal Antibody on Normal Neonatal Rat Islet Cell Cultures

Islet cell cultures were prepared and employed in a ^{51}Cr-release

assay as described previously. However, these cultures consisted of beta cell as well as many other cell types; for example, alpha, delta and pp cells, exocrine cells and fibroblasts. Therefore, the equation for determining toxicity (lysis of beta cells) was modified to take this into account:

% lysis = 100 X [(^{51}Cr release in the presence of KT1 + complement) - (^{51}Cr release with complement alone)]/[(^{51}Cr release in the presence of rabbit anti RINm5F sera + complement) - (^{51}Cr release with complement alone)].

K. Cloning of Hybridomas

Positive hybridomas were cloned by limiting dilution to produce hybridoma colonies secreting antibody of a single specificity. This involved adding 0.1 ml of hybridoma cell suspension containing 50, 20, 10 and 5 cells per ml to each well of 96 well tissue culture plates previously seeded with thymocyte feeder cells. When the hybridomas were semiconfluent the supernatants were assayed for cytotoxic ICSA again and positive clones were transferred into 24 well cluster plates (Linbro). At this stage the antibodies were analyzed by an Ouchterlony assay to determine which immunoglobulin subclass they belong to (Ouchterlony, 1949).

L. Subtyping

The isotype of the monoclonal antibody was determined by Ouchterlony double diffusion in agar using anti-rat Ig subtype-specific antibodies from Cappel Labs. In this assay, the central well was filled with 10 µl of the monoclonal antibody and the peripheral wells

were filled with 5 ul of either a 1:1 dilution or a 1:10 dilution of class-specific anti-rat-immunoglobulin antibodies (1 mg/ml active antibody). Diffusion was allowed to proceed at room temperature for 6 hours after which time precipitation lines had clearly formed.

M. Affinity Isolation of Monoclonal Autoantibody

Hybridoma supernatant was first concentrated by high pressure membrane dialysis using an Amicon filter with a 100,000 MW cut off size. A column containing 50 mls of agarose beads conjugated to anti-rat IgM (Hyclone Laboratories, Inc.) was washed in blocking serum prior to incubating the beads with the concentrated hybridoma supernatant for 2 hours. The column was eluted with 0.3M acetic acid containing 0.55% NaCl. The eluate was dialysed immediately against a high salt buffer containing 1.5 M NaCl pH 8.0. The protein solution was then concentrated by high pressure membrane dialysis and stored at $-70^{\circ}C$.

N. Protein Determination

The amount of protein obtained from the affinity column was estimated by a Biorad Colorimetric assay (Bradford Assay). In brief, 5 dilutions (0.2, 0.5, 0.8, 1.1 and 1.4 mg/ml) of the Biorad protein standard were prepared and 0.1 ml of each standard, the unknown and a sample buffer "blank" were placed in test tubes. Five ml of a 1:5 dilution of the dye reagent was added to each tube. After mixing by inversion the tubes were left for 10 minutes. The OD_{595} of each sample was measured against the reagent blank. The OD_{595} was plotted against

the protein concentration for each of the standards and the unknown was read from the standard curve.

O. Indirect Immunofluorescence of Monoclonal Antibody on Cells Grown On Coverslips

RINm5F cells and primary rat and mouse beta cells were cultured on glass coverslips. Indirect immunofluorescent staining was carried out on either live cells or cells fixed in 2% paraformaldehyde in 0.15 M PBS for 10 minutes followed by washing in 0.15 M PBS, containing 10% FCS, for 5 minutes. The coverslips were incubated for 30 minutes at 37^{o}C, with monoclonal antibody (Mab) (20 ug protein in purified IgM preparation or 200 ul undiluted hybridoma supernatant) washed in 3 changes of 0.15 M PBS containing 10% FCS, and then 200 ul of a 1/50 dilution of goat anti-rat IgM conjugated to Tetramethylrhodamine isothiocyanate (TRITC) (Cooper Biomedical) was added to each slide. After a 30 minute incubation at 37^{o}C, the slides were washed, mounted on to glass slides with orthophenylenediamine in buffered glycerol, and observed by epifluorescence with a Zeiss microscope using two BP546 nm interference filters.

In some experiments a biotinylated anti-rat IgM secondary antibody was used instead of anti-rat IgM-TRITC. In such experiments an additional step involving an incubation with TRITC-avidin was employed. In addition to working with cells grown onto coverslips, immunofluorescent staining was also performed on fixed, and on frozen sections of rat pancreas and on RINm5F cells in suspension.

P. Indirect Immunofluorescence of Monoclonal Antibody on Cell Suspensions

RINm5F cells were detached from culture flasks with PBS without Ca^{++} and Mg^{++}. To avoid degrading the antigen, trypsin was not used. The cells were washed twice in RPMI, (containing 5% FCS to block any non-specific binding). 10^6 RINm5F cells were dispensed into each Eppendorf tube and the cells were incubated in 200 ul of the monoclonal antibody containing 20 ug total protein or 200 ul undiluted hybridoma supernantant for 40 minutes at 4^oC. The cells were then washed three times and incubated for a further 40 minutes in 200 ul of anti-rat IgM conjugated to TRITC. After further washing in RPMI the cells were washed twice in PBS and transferred to glass slides. The slides were examined on a Zeiss microscope fitted with a light source and optics for immunofluorescence as described in section N.

Q. Western Blots

RINm5F cells were lysed with CHAPS (3-[(3-cholamidopropyl)dimethyl ammonio]^1 propanesulfonate, Sigma) in PBS (20 mM CHAPs containing 0.2 mM PMSF) and 100-300 ug/well total protein was loaded onto SDS polyacrylamide gels (4% stacking gel and 10% running gel, 1.5 mm thickness) and the gels were electrophoresed at 4^oC and 80 V overnight. Biorad SDS-PAGE molecular weight standards (MW range 10,000-100,000 and 40,000-250,000) were included on each gel. The protein bands were transblotted onto nitrocellulose (NC). In this procedure the gel was overlayed on the NC which was then sandwiched between 4 layers of filter paper and secured in a cassette. The cassette was immersed into precooled (4^oC) transfer buffer (25 mM Tris, pH 8.3, 150 mM glycine and

20% v/v methanol) and was run at 4°C and 100% voltage for 2 hours. The NC was then cut into strips and incubated with blocking buffer (3% BSA or 1% skimmed milk powder in TRIS-Saline) for 2 hours at room temperature. The nitrocellulose strips were then washed in TRIS-saline containing 0.005% TWEEN at room temperature and incubated overnight with antibody. Alternatively the NC strips were incubated overnight at 4°C with blocking buffer and 2 hours at 37°C with the monoclonal antibody (undiluted hybridoma supernatant or 40 ug/ml purified Mab). After thorough washing the NC strips were incubated with 1:200 dilution of peroxidase labeled anti-rat IgM (H and L, Hyclone, Utah) in a 3% BSA-TRIS saline solution for 2 hours. Following further washing the strips were developed in the horse raddish peroxidase substrate (60 mg 4-chloro-1-napthol in 20 mls methanol added to 50 ul 30% H_2O_2 in 100 mls PBS). The colour development was stopped by rinsing the NC in distilled H_2O which was followed by an overnight wash in distilled H_2O.

RESULTS

To date, no conclusive data are available on whether there is a correlation between the presence of cytotoxic ICSA and diabetes in the BB rat. In the present study, the association between the presence of ICSA and the development of overt diabetes was examined in 65 BB rats. The rats were followed from 60-150 days old during which time they were monitored for overt diabetes and cytotoxic ICSA. Any sample giving a values above 5.2% in the microcytotoxicity assay was considered to be positive for cytolytic ICSA activity. This value was calculated from the mean + 3 SD of a random sample of twenty negative observations extracted from the BB rat data.

I. Cytotoxic ICSA In Vivo

A. Correlation between cytotoxic ICSA and diabetes: In terms of the relationship between ICSA and diabetes, the rats appear to fall into four categories (Figure 1).

Diabetic rats: Among the 33 diabetic animals, 58% exhibited ICSA by 150 days; accordingly 29% of the total population exhibited diabetes and ICSA (Figure 1). ICSA was present in 7 rats prior to, and in 12 rats after, the onset of overt diabetes. Twenty-two percent of the rats were overtly diabetic without showing any detectable ICSA (Figure 1).

Non-diabetic rats: Fourteen rats (22%) had positive sera but did not become diabetic within the time period studied whereas 18 rats (28%) exhibited neither ICSA nor diabetes. A previous study demonstrated that 85% of BB rats which are going to become diabetic do

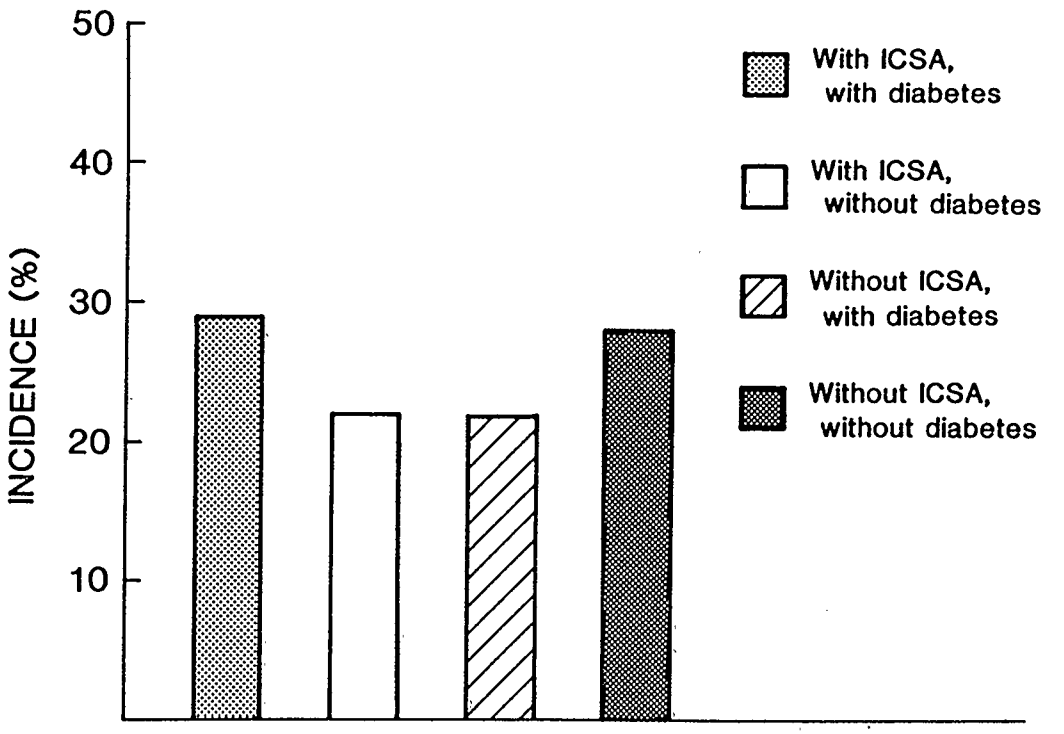


Figure 1: Incidence of ICSA and diabetes in the total BB rat population calculated from data derived from 65 BB rats assessed for cytotoxic ICSA and overt diabetes as described in the Materials and Methods.

Table 1: Presence of ICSA and/or Diabetes in BB Rats

a*	+diabetes/+ICSA		+diabetes/-ICSA		-diabetes/+ICSA		-diabetes/-ICSA	
	N°	%	N°	%	N°	%	N°	%
BB_o	8	28	8	28	4	14	9	31
BB/W	11	31	6	17	10	28	9	25
b**								
male	3	25	1	8	3	25	5	42
female	5	29	7	41	1	6	4	24

* Total BB rat population grouped according to source.

** Comprised of the BB_o colony grouped according to gender.

so by 120 days old (Like et al., 1982a). Therefore, most of the rats in the present study were followed to at least 140 days old. In the present study 24% of the diabetic BB rats became diabetic after 120 days old. Statistical analysis demonstrated that there was no association between cytotoxic ICSA and overt diabetes in the total rat population (X^2 test). The incidence rate of diabetes was 51% in the total population (n = 65) and the average age of onset was 108 $\pm$ 29 days (mean $\pm$ SD). Cytotoxic ICSA were present in 51% of the rats and the average age at which the autoantibodies were initially detected was 115 $\pm$ 28 days.

B. Genetic factors: Since BB rats from two different sources were used in this study a comparison of the results obtained from each subline was made (Table 1a). The BB_o population was found to have a higher incidence of diabetes (55%) than the BB/W population (47%). On the contrary, 58% of the BB/W population exhibited ICSA whereas only 41% of the BB_o population showed ICSA. Twenty-eight percent (10/36) of the BB/W rats, as opposed to 14% (4/29) of the BB_o rats, had ICSA but did not get diabetes. On the other hand, only 17% (6/36) of the BB/W, as compared to 28% (8/29) of the BB_o have diabetes but not ICSA (Table 1a). There was found to be no significant correlation between cytotoxic ICSA and overt diabetes in either subline (Fischer exact test).

Of the 8 BB_o rats which exhibited both cytotoxic ICSA and overt diabetes, antibody was detected prior to the onset of diabetes in 3 of them (Fig. 2a) and the other 5 rats exhibited detectable levels of antibody at or after the onset of diabetes (Fig. 2b).

C. Longitudinal study: When the occurrence of diabetes and cytotoxic

Figure 2: Cytolytic activity against RINm5F cells of sera from 8 BB_o rats which exhibited both cytotoxic ICSA and overt diabetes. The rats were bled sequentially approximately every 7 days from a young age up to 1-6 weeks after overt diabetes (day 0). Values were derived from duplicate determinations. Different symbols represent individual rats.

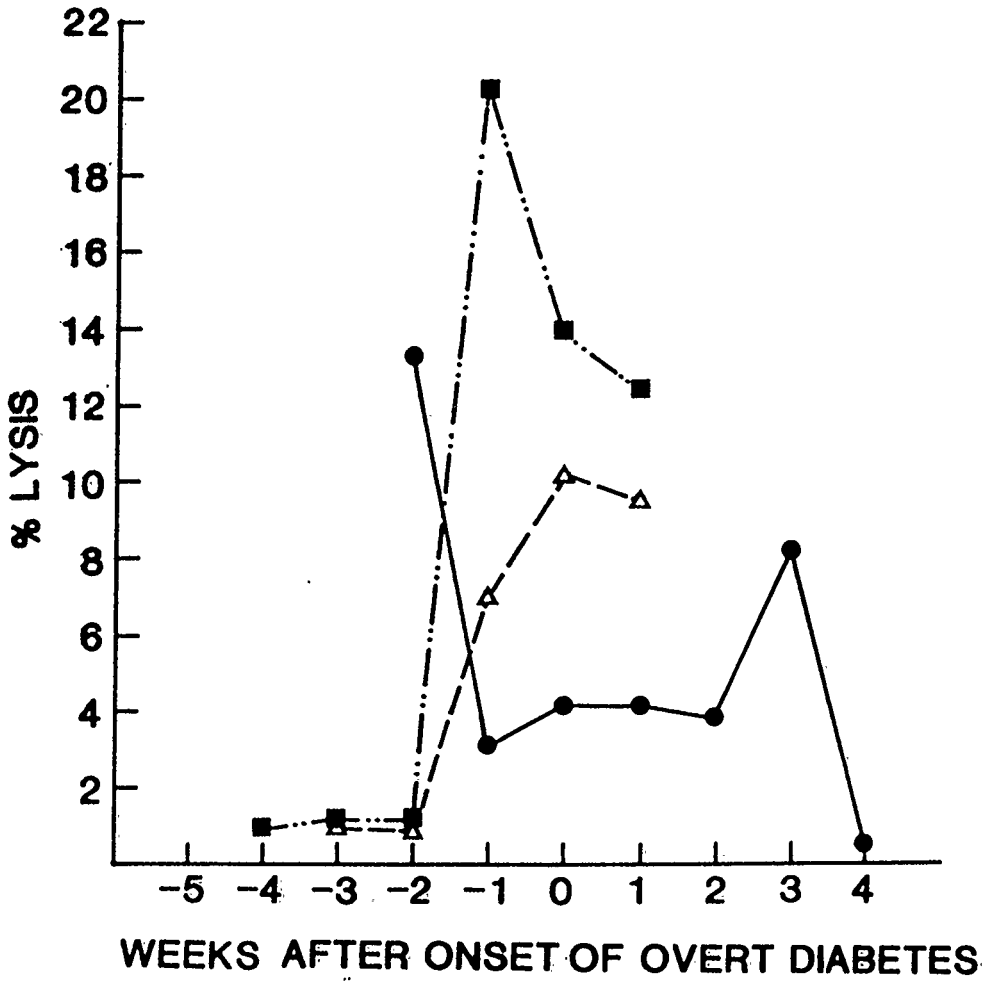


Fig. 2a: antibody was detected prior to the onset of diabetes in 3 BB_o rats.

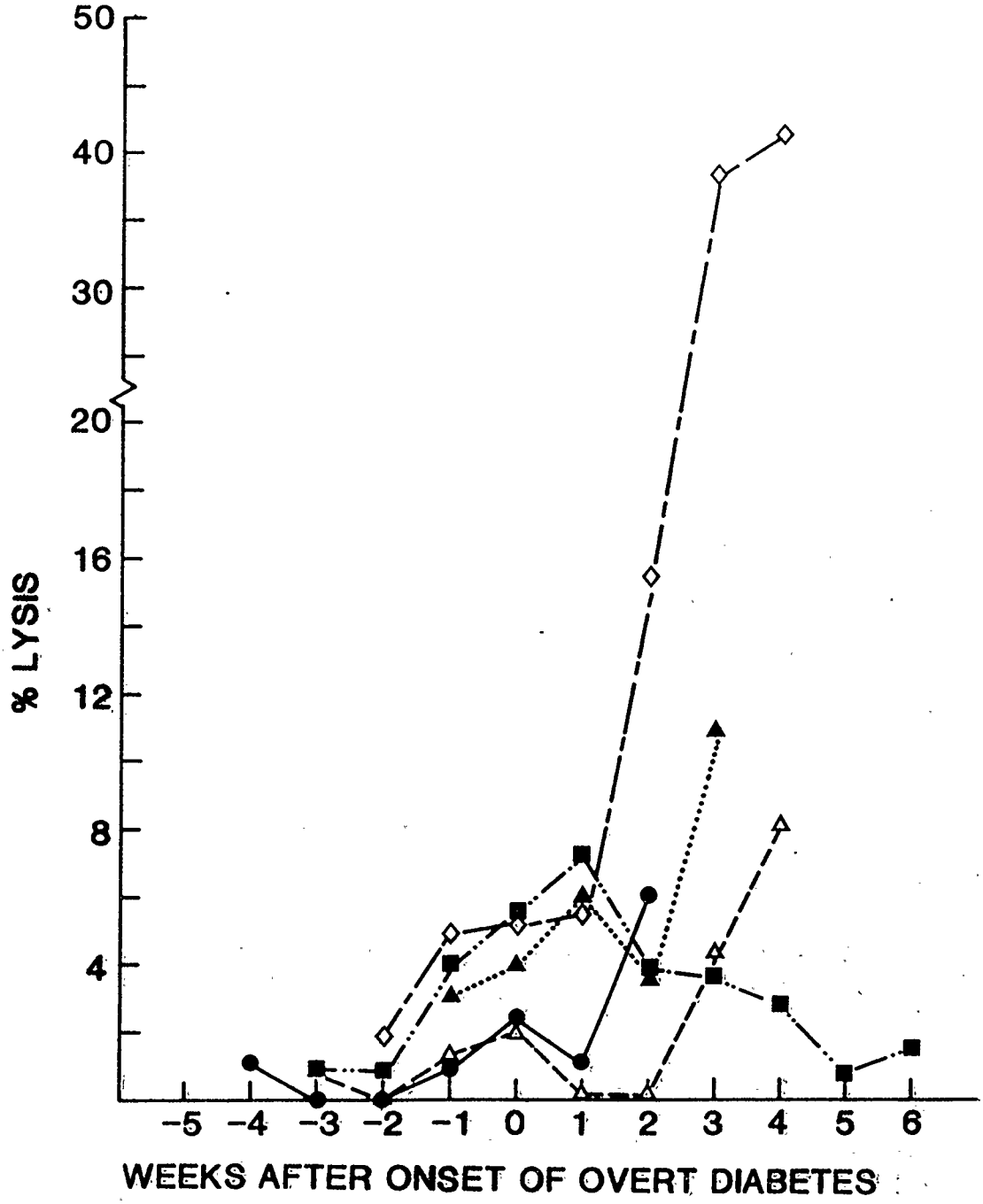


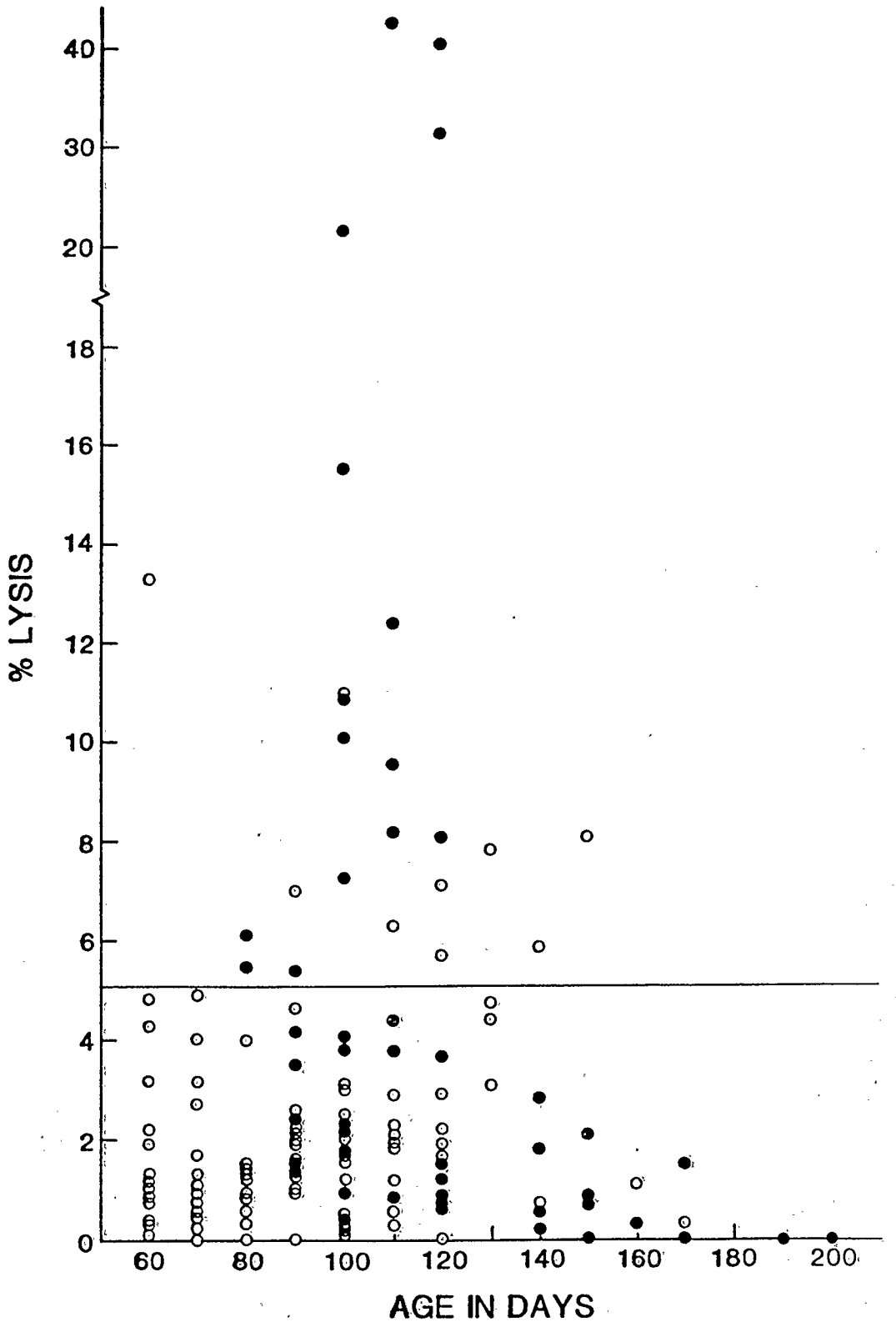
Fig. 2b: antibody was detected at or after the onset of diabetes in 5 BB_o rats.

ICSA in the two BB rat populations was followed over time (Fig. 3 a and b), no clear cut pattern emerged although in both populations a peak of observations with high levels of ICSA was detectable around 100 to 120 days old. These can partly be accounted for in the BB_o subline by 2-3 rats which showed unusually high titres of ICSA all of which were diabetic. Points indicating overt diabetes are scattered evenly throughout the observations although as expected the number of diabetic observations increase with time. A larger proportion of diabetics than nondiabetics, however, do occur among the ICSA positive observations. In general these results support the other findings which have been presented in this study.

D. Gender factors: The BB_o population consists of 17 female and 12 male rats. The occurrence of ICSA and diabetes was compared between the males and females within this group (Table 1b). The incidence of diabetes in the male population was 33% as opposed to 71% in the female population. Thirty-five percent of females compared with 50% of males were ICSA positive. In addition, females exhibited ICSA at an earlier age (86 ± 14 days) than males (113 ± 23 days) although this was not significant. Many more females than males became diabetic without showing positive sera. However, statistical analysis of the above data showed that there was not a significant correlation between ICSA and diabetes in the male or the female rats (Fischer exact test).

E. Induction of ICSA: To determine if stimulation of ICSA production can induce diabetes, 7 BB rats were injected with 10^6 RINm5F cells intraperitoneally. The rats were bled periodically and their sera assayed for cytotoxic ICSA. Six of the rats produced ICSA of a higher titer than untreated rats and 3 of these rats developed diabetes within

Figure 3: Cytotoxic activity against RINm5F cells of sera from 29 BB_o rats (Fig. 3a) and 36 BB/W rats (Fig. 3b). The rats were bled sequentially from an early age. Serum samples resulting in cytotoxic ICSA activity above the mean + 3 SD of control sera (see text) were considered positive (indicated by solid line). Values were derived from the mean of duplicate determination. Variations from the reported mean was less than 10%. ● represents diabetic rats and ○ represents non-diabetic rats.


% LYSIS
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4
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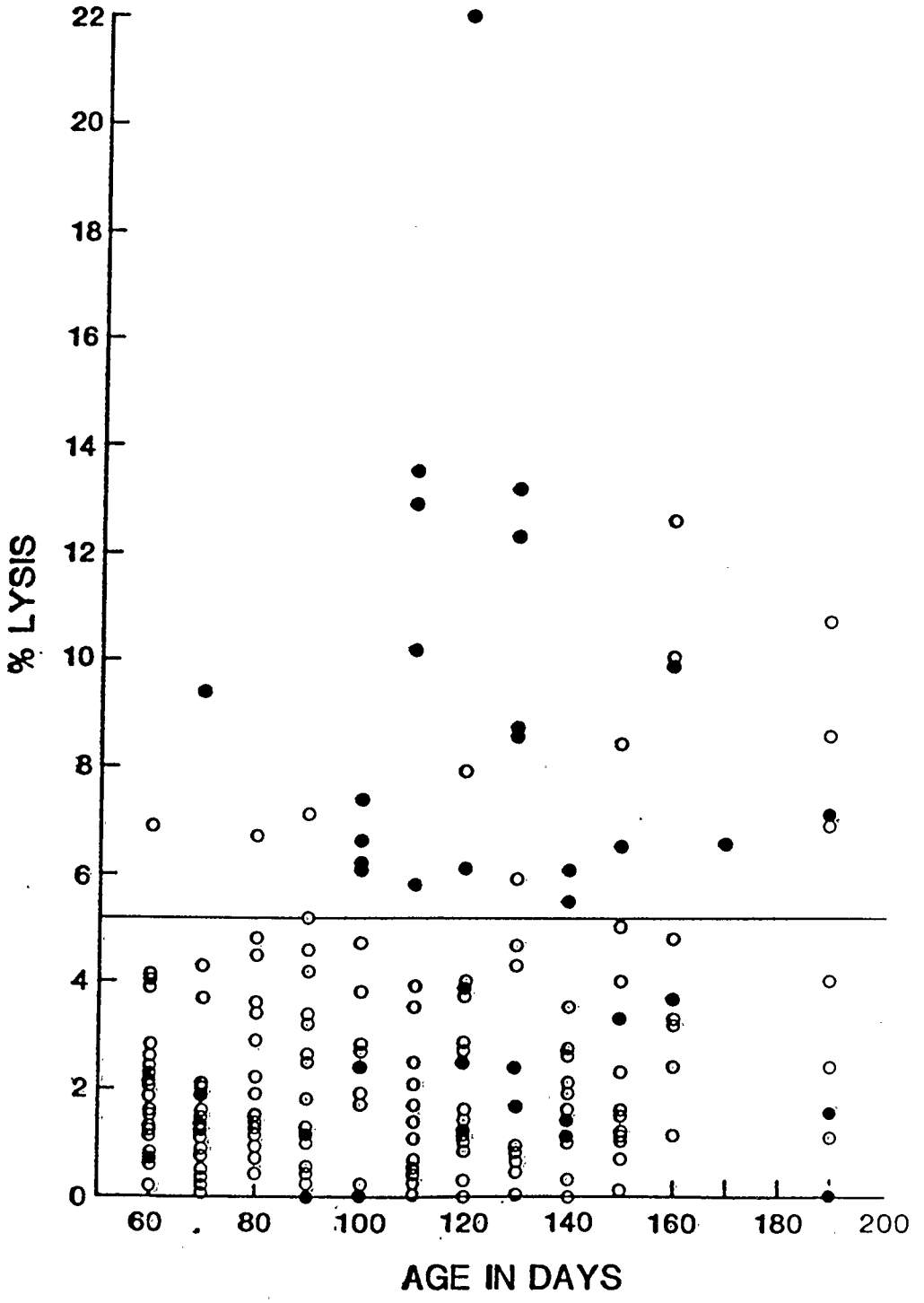
Figure 3a


22
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200
AGE IN DAYS

Figure 3b

Table 2

Presence of ICSA and/or Diabetes in 7 BB Rats Which Have Previously Been Injected With RINm5F Cells*

	+Diabetes	-Diabetes
+ICSA	3	3
-ICSA	1	0

* Animals were injected with 10^6 RINm5F cells i.p. or i.v. at approximately 90 days of age. They were then bled at weekly intervals and tested for cytotoxic ICSA. Diabetes was monitored by biweekly glycosurias. Animals were followed for 4-5 weeks after injection.

a 40 day period following the injection (see Table 2). However, the other 3 rats with ICSA positive sera did not develop diabetes and one rat which failed to elicit an antibody response did become diabetic.

II. Cytotoxic ICSA In Vitro

A. Generation of monoclonal ICSA: The BB rats were screened for the presence of cytotoxic ICSA and the rats exhibiting over 6% lysis in a microcytotoxicity assay were killed and their spleen cells fused with myeloma cells. Antibody positive, prediabetic and diabetic rats were used in the fusion and their ages ranged from 70 to 200 days. More than forty fusions were performed resulting in over six thousand hybridoma clones. Fusion efficiency was high especially in the fusions where rat spleens were fused with the mouse myeloma cell lines (50% fusion efficiency). However, only one useful hybridoma was obtained using the microcytotoxic assay screening criteria. The protocol used to prepare the BB rat from which the aforementioned hybridoma came from was as follows:

Day 1 - bled from retroorbital sinus of BB rat 9/2

Day 2 - sera screened by microcytotoxicity assay exhibited 12.3% lysis of RINm5F cells (+ve control 28.5%)

Day 3 - injected 10^6 RIN cells into tail vein

Day 8 - BB rat bled

Day 9 - sera screened by microcytotoxic assay exhibited 38.3% lysis of RIN cells (+ve control 44.5%)

Day 10 - fusion between spleen cells of BB rat 9/2 and SP2 mouse myeloma cells

This hybridoma, produced from the fusion of spleen cells from a diabetic BB rat and the mouse myeloma cell line SP2/0, was subcloned three times by limiting dilution.

The ^{51}Cr-release assay was used to select the clone producing the highest cytolytic activity. In each case a clone was selected which exhibited approximately 50% of the positive control. The results of these assays are as follows:

1. Screened clones by microcytotoxic assay

Results:	Treatment	% Lysis
	+ve control	26.8
	-ve control	-1.7
	hybridoma well KT1 DC3	13.6%

Subcloned by limiting dilution.

Screened subclone 1 by microcytotoxic assay

Results:	Treatment	% Lysis
	+ve control	73.9
	-ve control	-5.1
	hybridoma well KT1 DC3FD9	44.0

Subcloned by limiting dilution.

Screened subclone 2 by microcytotoxic assay

Results:	Treatment	% Lysis
	+ve control	59.5
	-ve control	-7.1
	hyb snt KT1 DC3FD9NF9	28.7

B. Determination of isotype of Mab KT1: The monoclonal antibody, designated KT1, was found to be of the IgM isotype (Fig. 4).

C. Microcytotoxic assay on RINm5F cells: In microcytotoxic assays KT1

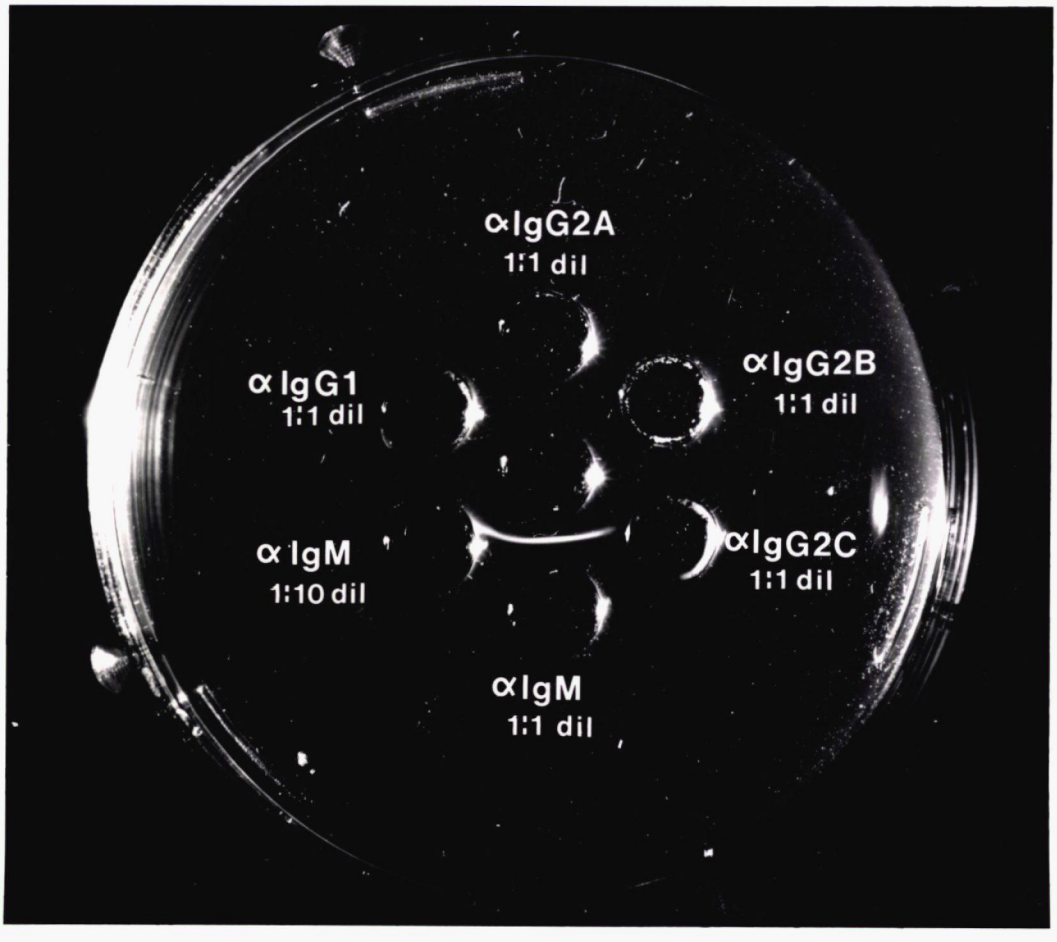

Figure 4: Ouchterlony assay to determine the isotype of the monoclonal antibody KT1

Table 3: Lysis of Various Types of ^{51}Cr-labeled Cells To Determine Specificity of MabKT1

	Percent Specific Lysis[a]					
	RINm5F	IEC-18	L-2	Vero	BHK	MKD
KT1 hybridoma supernatant	28.7	0	0	0	0	0
rabbit anti-RINm5F sera	62.0	26.8	20.4	2.8	50.0	0
nonspecific monoclonal antibody	0	0	0	3.7	0.5	0.9
ICSA positive BB rat serum	20.4	3.0	5.3	23.8	7.6	0

[a] Values represent the mean of duplicate evaluations. Variation from the reported means was less than 10%.

was found to mediate complement-dependent lysis of approximately 30% of a rat insulinoma cell line (RINm5F). However the Mab did not cause lysis of BHK, MKD, L-2 Vero or IEC18 (Table 3). The positive control, which consisted of an antisera, produced in rabbits against RINm5F cells, caused 62% lysis of the target cells.

D. Microcytotoxic assay of Mab KT1 on normal neonatal rat islet cell cultures: Mab KT1 was demonstrated to be cytotoxic to normal rat islet cell cultures in a ^{51}Cr-release assay. Rabbit anti RINm5F sera was taken to represent 100% lysis of beta cells in the islet cultures since these cultures were composed of many other cell types in addition to beta cells. Purified KT1 was found to cause 44.7% lysis whereas a 10 fold concentrated KT1 hybridoma supernatant brought about 26.3% lysis. A negative control, which consisted of an irrelevant rat monoclonal antibody of unknown specificity, caused 0% lysis.

E. Isolation of MAB KT1 by affinity chromatography: Mab KT1 was purified from hybridoma supernatant by a monoclonal antibody affinity isolation system consisting of anti-rat IgM conjugated to agarose beads. The first isolation obtained 3.2 mg total protein and the second isolation 1.3 mg total protein as determined by a Biorad colorimetric assay.

F. Indirect immunofluorescence of Mab KT1: The Mab KT1 was found to bind to a cell surface antigen on live (Fig. 5 and 6) and fixed islet cell cultures from Wistar rat neonates by indirect immunofluorescence. Negative controls using an irrelevant monoclonal antibody (mouse anti-frog retina antibody) or Wistar rat sera as second antibody did not show any staining. In addition, KT1 was also found to stain fixed mouse islet cell cultures (Fig. 7) and RINm5F cells (Fig. 8).

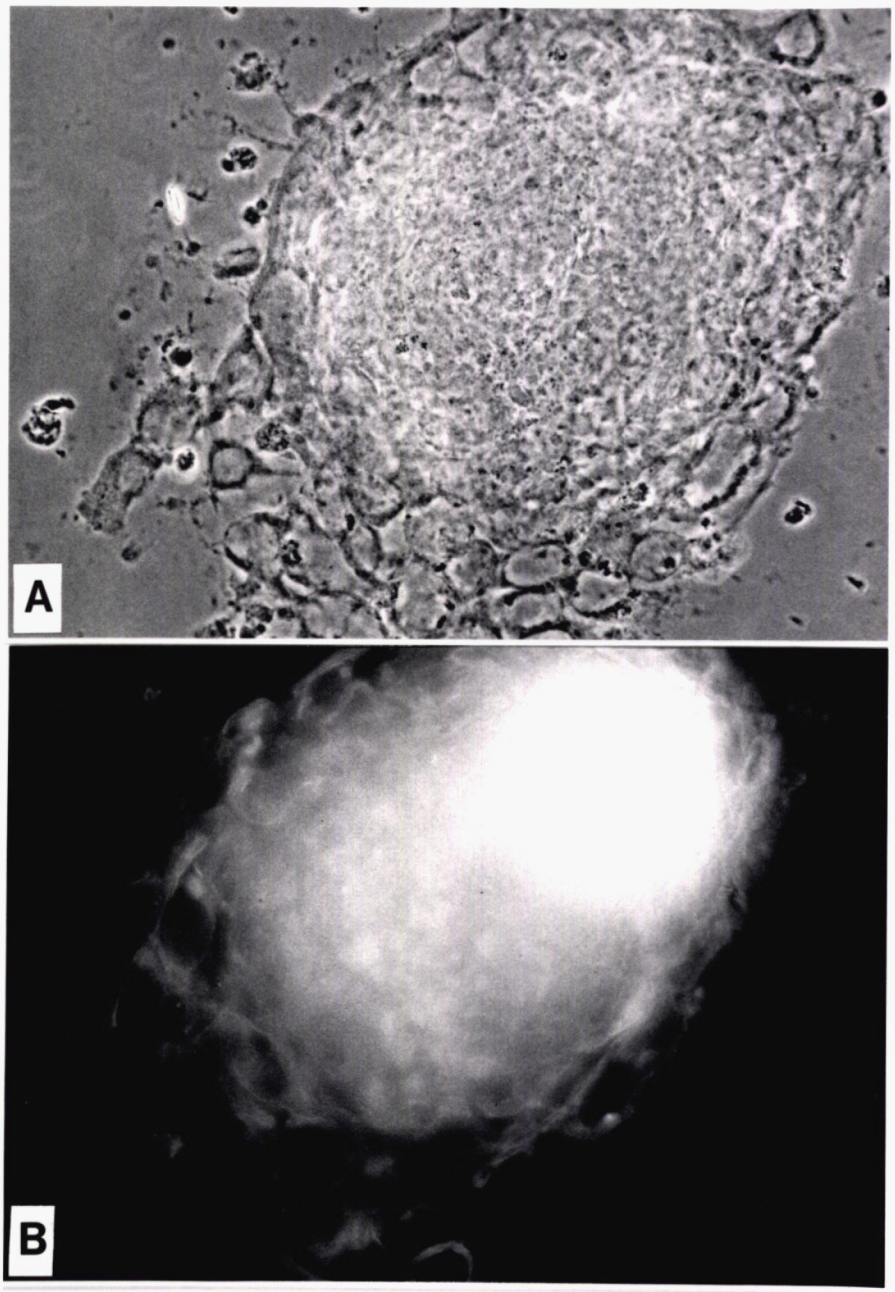

Figure 5: Indirect immunofluorescent staining of normal rat islet cell cultures incubated with the purified monoclonal antibody KT1 followed by TRITC conjugated to goat anti-rat IgM. a. phase contrast photomicrograph. b. fluorescent photomicrograph (X400).

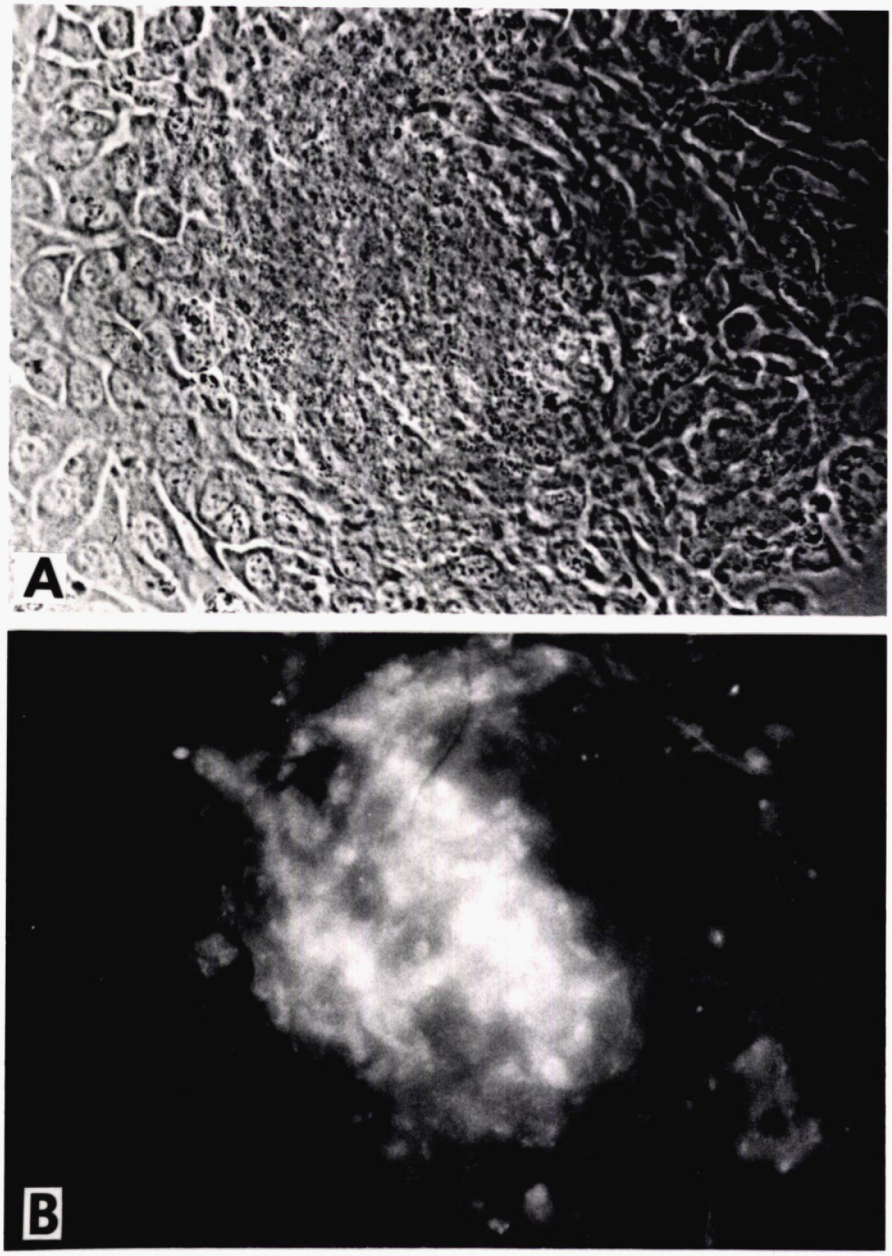

Figure 6: Indirect immunofluorescent staining of normal rat islet cell cultures incubated with the purified monoclonal antibody KT1 followed by TRITC conjugated to goat anti-rat IgM. a. phase contrast photomicrograph. b. fluorescent photomicrograph (X400).

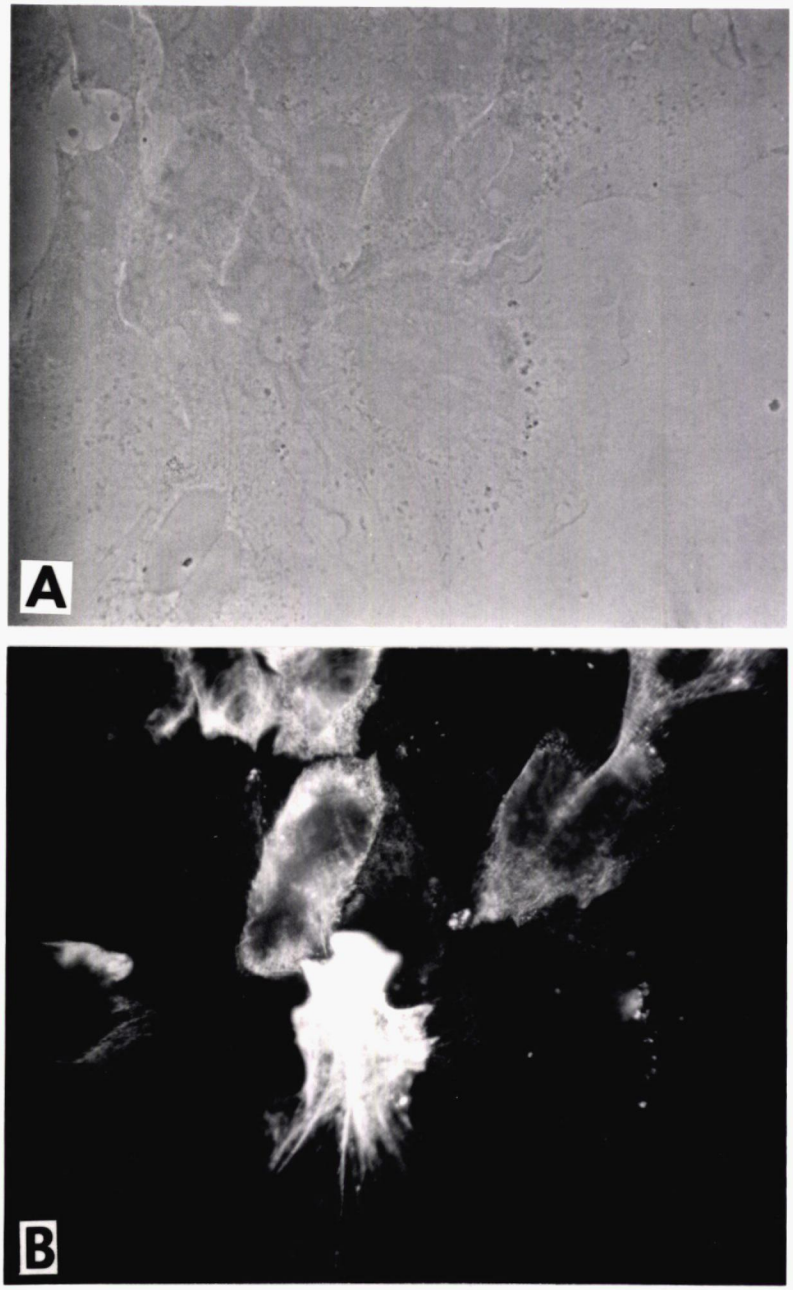

Figure 7: Indirect immunofluorescent staining of 2% paraformaldehyde fixed mouse islet cell cultures incubated with purified monoclonal antibody KT1 followed by TRITC conjugated to goat anti-rat IgM. a. phase contrast photomicroph. b. fluorescent photomicrograph (X1000).

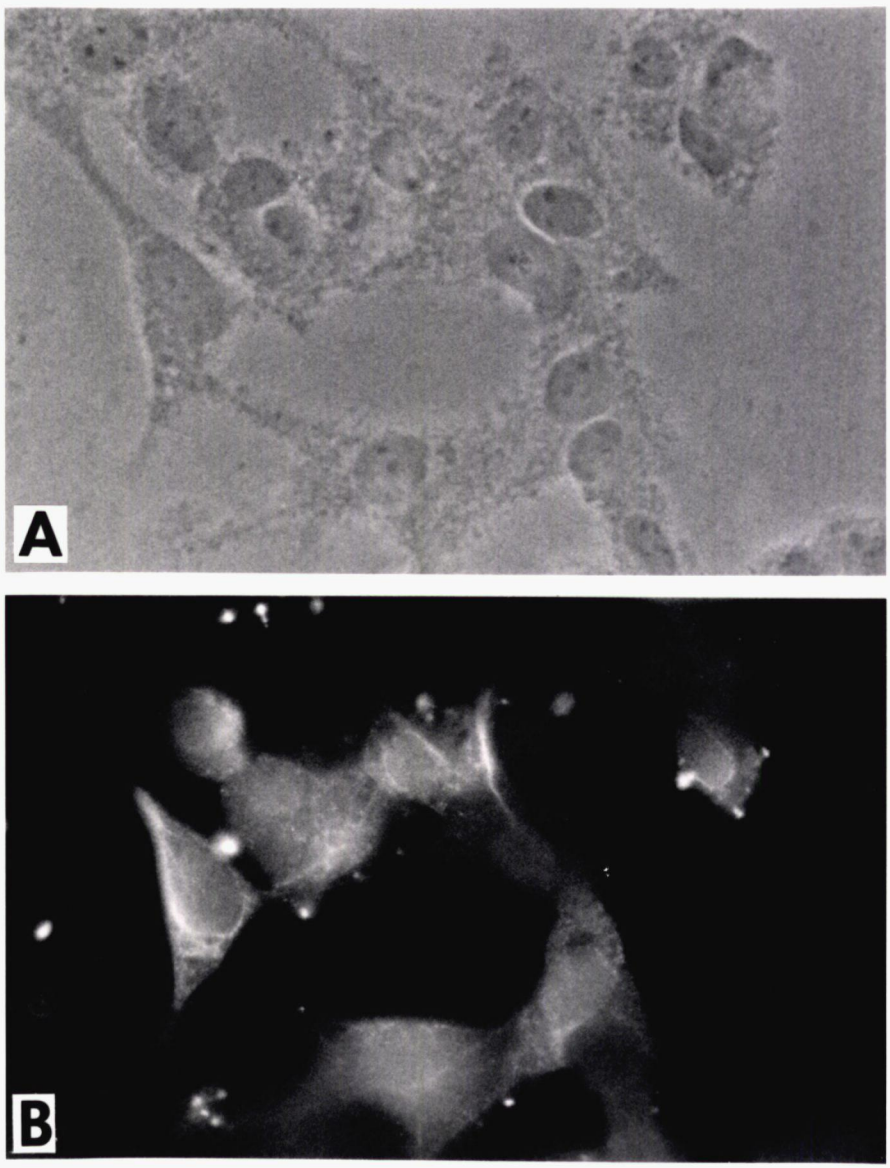

Figure 8: Indirect immunofluorescent staining of RINm5F cells incubated wtih purified KT1 followed by TRITC conjugated to goat anti-rat IgM. a. phase contrast photomicrograph. b. fluorescent photomicrograph (X1000).

Table 4

Indirect Immunofluorescence of Monoclonal Antibody KT1 on RINm5F Cell Suspensions

Sample Number	Treatment		Immunofluorescent Staining*
	1st Incubation	2nd Incubation	
1	Purified KT1	TRITC anti rat IgM	++++
2	Purified KT1	No antibody	+
3	No Antibody	TRITC anti rat IgM	+
4	+ ve BB rat sera	TRITC anti rat Ig poly	+++++
5	Conc hybridoma supernatant	TRITC anti rat IgM	++++
6	Wistar/Furth rat sera	TRITC anti rat Ig poly	++

* + Very Faint Immunofluorescence
++ Faint immunofluorescence
+++ Bright Immunofluorescence
++++ Very Bright Immunofluorescence
+++++ Brilliant Immunofluorescence

Moreover, KT1 was found to produce bright immunofluorescent staining on RINm5F cells in suspension (Table 4).

G. Western blot analysis: Extracts of RINm5F cells were subjected to SDS-PAGE and Western blotting. The results of immunostaining such blots can be seen in Figure 6. KT1 specifically stained one band of molecular weight 68,000d (left lane) BB rat sera, however, did not specifically stain any bands (middle lane). The sera used in this blot had been thawed for some time so the activity was probably very low. Supernatant from the myeloma fusion partner provided the negative control (right lane). Due to the instability of the rat-mouse hybridoma cells the monoclonal antibody KT1 is secreted by the clones for only a few cell generations and therefore it has not been possible to further characterize either the monoclonal antibody or the autoantigen.

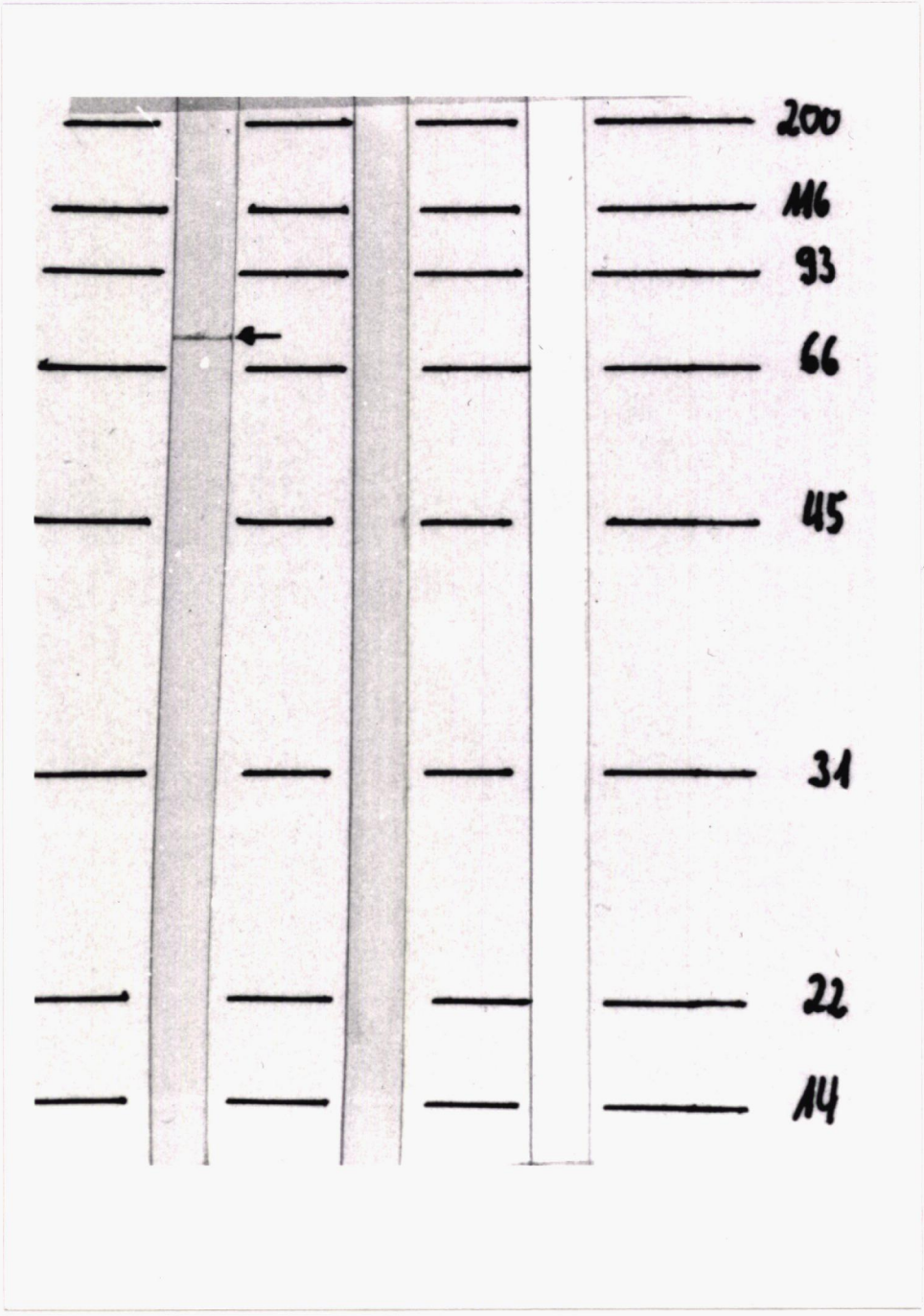

Figure 9: Western blot analysis of the binding of monoclonal antibody KT1 (left lane) to a RINm5F cell extract. Each lane contained 100 ug of protein. The middle lane represents BB rat sera and the right lane represents the supernatant of the myeloma fusion partner.

DISCUSSION

There is little doubt that autoimmunity plays a principal role in the etiology of IDDM. As has been shown, there are many lines of evidence to support the hypothesis of an autoimmune pathogenesis of diabetes in both the BB rat and man. However, the effector cell(s) responsible for beta cell destruction is still not known. Indeed, there are so many immunological abnormalities and autoimmune phenomena associated with diabetes that it is difficult to determine which are the major culprits.

At the present time, the potential role of circulating islet cell antibodies in IDDM is conjectural. This is partly because autoantibodies can be found in the serum and tissues of a remarkably large number of apparently normal individuals, particularly in older age groups. Apparently innocuous autoantibodies are also formed following damage to tissue and, according to some reports (reviewed by Graber, 1975), these serve a physiological role as carriers of the products from tissue breakdown. Antibodies directed toward cytoplasmic islet cell antigens are unlikely to initiate immune damage in pancreatic islets, rather, they may serve to opsonize previously damaged tissue for removal by macrophages. Thus, ICA are probably an epiphenomenon, associated with the onset of IDDM but not the cause of the disease. Islet cell surface antibodies, however, are more likely candidates in the immunopathogenic mechanism(s). Many of the properties exhibited by ICSA make them possible contenders for initiating beta cell destruction.

Cytotoxicity would be considered as an important criterion for a primary mediator of disease and as such ICSA have been demonstrated to cause specific beta cell damage in vitro, although this remains to be shown in vivo. Ideally, at least three requirements should be met before a disorder can be categorized as being truly due to autoimmunity: 1) the presence of an autoimmune reaction, 2) the absence of another well-defined cause of the disease and 3) clinical or experimental evidence that such a reaction is not secondary to tissue damage but is of primary pathogenetic significance. The first two of these criteria are applicable to IDDM. However, the third still has to be addressed.

This thesis attempts to examine the possibility that humoral autoimmunity is of primary pathogenetic significance in IDDM. It is not known to what extent the appearance of ICSA signifies islet cell destruction. One way to evaluate the role of ICSA in the pathogenesis of IDDM is to determine if there is a close correlation between cytotoxic ICSA and overt diabetes in an appropriate animal 'model'. The BB rat is probably the most important animal 'model' for IDDM, since the diabetes exhibited by these animals has many similarities to human IDDM. For this reason BB rats were followed in this study and the onset of overt diabetes together with the detection of cytotoxic ICSA were monitored over time.

These results suggest that expression of cytotoxic ICSA is not a prerequisite for overt diabetes in the BB rat, nor does the presence of cytotoxic ICSA necessarily indicate that diabetes will ensue. However, the possibility that some degree of beta cell destruction occurred cannot be excluded. Nevertheless, these results imply a lack of

correlation between cytotoxic ICSA and overt diabetes. If this is the case, it may be possible to genetically segregate the determinants for ICSA production and diabetes. Although this was not attempted in this study, it is interesting to note that the frequency of ICSA between rats from two different sources varied considerably. There was a 2:1 ratio of BB_o:BB/W rats developing diabetes but not the antibody. In contrast there was a 1:2 ratio of BB_o:BB/W rats showing the antibody but not the disease. This suggests that the two populations which were originally from the same colony, may have drifted genetically with regard to these particular characteristics. The concept that genetic control of ICSA production may be disassociated from diabetes is not new. In a study involving 111 patients with IDDM and all their first degree relatives Toguchi and coworkers (1985) found an increased prevalence of ICSA in unaffected siblings of ICSA-positive probands (33%, 17/52) but not in those of ICSA-negative probands (5%, 6/117) ($p < 0.001$). This finding supports the proposal that ICSA and diabetes may be genetically independent. The expression of ICSA may be heritable and those individuals in whom ICSA is associated with diabetes may "differ" from those who are diabetic without exhibiting detectable levels of ICSA. Some workers (Morris et al., 1976; Christy et al., 1976; Irvine et al., 1977) have reported that insulin dependent diabetics with the HLA-B8 haplotype are more likely to exhibit autoantibodies to pancreatic islet cells than patients who are lacking this antigen. Furthermore, it has previously been suggested that the occurrence of IDDM may be associated with a familial predisposition to autoimmunity in some patients but not in others (Nissley et al., 1973; Lendrum et al., 1976, Barbosa et al., 1982). The heterogeneity found

in BB rats, therefore, may represent yet another similarity to human IDDM. Moreover, the variation between populations could be exploited to reveal important information about the various autoimmune phenomena associated with diabetes.

In any event, caution should be applied when interpreting and comparing results from different BB rat colonies since variation appears to exist not only in the incidence of diabetes and the frequency of ICSA but also in the association of the antibody with the disease.

Another approach to assess the importance of ICSA in the disease pathogenesis is to determine if ICSA develop prior to the clinical onset of diabetes. In the present study the majority of the BB rats, which manifested both autoantibody and disease, expressed ICSA after the onset of overt diabetes. Furthermore, the mean age at the onset of diabetes in the total population was younger when compared to the mean age that ICSA was first detected, although this was found not to be significant by a students t-test. When the levels of cytotoxic ICSA in the BB_o subpopulation were examined over time, only 3 rats were found to exhibit an ICSA peak prior to disease onset (Fig. 2a). Even then the peak occurred within 2 to 3 weeks of the ensuing diabetes. On the other hand, 5 BB rats exhibited peak levels of ICSA after the onset of overt diabetes (Fig. 2b). These animals were negative for ICSA prior to disease onset. Taken together, these results would imply that the autoantibody was more likely to be a result of the disease than a cause of the disease. The lack of correlation between cytotoxic ICSA and overt diabetes is further supported by the data from a longitudinal study of the two BB rat subpopulations. In both the BB_o (Fig. 3a) and

the BB/W (Fig. 3b) rats, observations of diabetes are distributed throughout the graph in both the presence and absence of ICSA. Although there is a definite trend toward diabetes in the ICSA positive observations, when the mean cytotoxic values during the observation period were compared there are no significant differences between the population of rats with overt diabetes and the population of rats which did not get diabetes. Some studies have suggested a closer correlation between the appearance of ICSA and the onset of diabetes in the BB rat (Martin and Logothetopoulos, 1984; Baekkeskov et al., 1984) whereas others have lent support to the present findings (Pollard et al., 1983; Dyrberg et al., 1984; Dean et al., 1987). These studies will be discussed in more detail later.

It is currently accepted that male and female BB rats develop diabetes with equal frequency (Nakhooda et al., 1976; Rossini et al., 1983). However, in the present study diabetes was more frequent in female rats (12/17) than in male rats (4/12) (Table 1b) which is difficult to explain. The work generally cited describing the lack of sex-related differences in the incidence of diabetes is the original studies carried out by Nakhooda and coworkers (1976, 1978). They found 9 out of 51 BB rats developed diabetes, 5 out of the 24 females and 4 out of the 27 males. More recent data have been provided by Rossini and coworkers (1983) who found that castration had no significant effect on the incidence of diabetes. In their control population 57% (4/7) males and 43% (3/7) females had diabetes. These incidences changed only slightly following castration. It is difficult to draw conclusions from either of these examples due to the small number of rats used and it is possible that had the sample size been larger in

the present study, a more even distribution of diabetes between males and females would have been found. Interestingly, however, some studies on human IDDM have shown a slight preponderance (12-20%) of males among younger diabetic children (Creutzfeldt et al., 1976; Nerup et al., 1980; La Porte et al., 1981) and in the NOD mouse model of IDDM a gender related difference in the incidence of diabetes is well established. The cumulative incidence of diabetes in females reaches 80% by 30 weeks whereas only 10% of males exhibit diabetes by 30 weeks in this mouse 'model'.

Our finding that more male rats had positive sera than the females was surprising since one would expect the opposite to be the case. Females are normally more susceptible to autoimmune phenomena than males. However the females in the present study did appear to exhibit ICSA at an earlier age than males. Gender-related differences in the presence of ICSA have also been reported in humans (Toguchi et al, 1985). In a study involving 98 probands, female relatives were more often ICSA positive than males. Forty-three percent of sisters compared with 21% of brothers of ICSA positive probands exhibited ICSA. Mothers are also more likely than fathers to be ICSA positive. Nevertheless, the numbers of rats involved in the present study are small and the difference in ICSA positivity (6/17 females as opposed to 6/12 males) is probably too slight to be significant.

Taken together the previously discussed results suggest that spontaneously produced cytotoxic ICSA alone are not responsible for the development of overt diabetes in these BB rats. To further test this hypothesis ICSA were induced in the BB rats and the effects of these antibodies on diabetes was measured. The resulting incidence of

diabetes in the rats which developed an elevated cytotoxic ICSA response was not significantly different from the untreated population. It is possible that these rats did incur islet cell damage but this was not examined. However, it appears that, even when cytotoxic ICSA are artificially induced in higher titers than those normally observed, overt diabetes does not necessarily follow.

Contradicting results have been provided by previous studies which have examined the relationship between ICSA and diabetes. A great deal of variation in the levels of ICSA were found to exist between different studies and indeed within and between individual rats. As discussed in the Introduction (pp. 34-35) a number of workers have not found a close correlation between ICSA and diabetes or indeed glucose intolerance and insulitis in their BB rat colonies (Dyrberg et al., 1984; Dean et al., 1987). On the other hand, in some reports a correlation does appear to exist (Baekkeskov et al., 1982; Martin and Logothetopolous, 1984).

Wide divergencies in the frequencies of ICSA between studies on diabetic patients and their relatives have also been reported. Some of the discrepancies are shown in Tables 5 and 6 which list the studies on the frequency of ICSA in patients with IDDM and in control subjects. The incidence of diabetic patients with ICSA positive sera range from as low as 32% to as high as 94%. Some of this variability can be accounted for by the differences in interpretation of the terms "recent onset" or "newly diagnosed". Whereas some workers measure for ICSA in patients with the onset of diabetes occurring within the last 6 months, others regard recent onset to be within the last 2 years. It is well documented that ICSA decrease with increasing duration of diabetes and

therefore the frequency of ICSA will be lower in the studies on patients with disease of longer duration. Another factor which will add to the variability between studies on BB rats as well as humans is the sensitivity of the assay system used. Most commonly used are indirect immunofluorescence and ^{125}I-protein A to measure binding of ICSA to viable or fixed islet cells or RINm5F cells. Cytotoxic ICSA are usually measured by quantifying beta cell death after exposure to sera by a ^{51}Cr-release assay, as in the present study, or by ethidium bromide or trypan blue exclusion assays.

It is inevitable, with the different techniques and different substrates employed that the results will not necessarily agree. Moreover, subjective evaluation will come into play to further skew the results. For example, in the present study ICSA positivity was set at observations which were above the mean + 3SD of a random sample of negative controls. In the literature the criteria varies from the mean + 2SD to the mean + 4SD.

However, even allowing for the variability in ICSA frequency brought about by these factors there is too great a range in the values to be solely due to methodology. Perhaps, real differences in ICSA positivity do occur between populations. This interpretation of the data supplied in Tables 5 and 6 would agree with the present findings of diversity between the two subpopulations of BB rats. This would, therefore, lend support to the hypothesis that the expression of ICSA is heritable and segregation of the determinants for ICSA production and diabetes may be possible.

The data provided in these Tables also supports the present finding that the onset of IDDM does not appear to be dependent on the

TABLE 5

ICSA in Patients with IDDM and Control Subjects

Substrate	Patients with IDDM %	Patients with IDDM Positive/ Tested	Control Subjects %	Control Subjects Positive/ Tested	Reference
Cultured Human Insulinoma[b]	87.2	34/39	3.3	1/30	Maclaren et al. (1975)
Rat Islet Cells[b]	31.8	28/88	4.2	1/24	Lernmark et al. (1978)
Rat or Mice Islet Cells[b]	66.6	22/33	2.7	2/74	Lernmark et al. (1981)
Human Fetal Islet Cells[b]	81.8	9/11	20.0	2/10	Pujol-Borrell et al. (1982)
Rat Islet Cells[b]	93.8	15/16	5.6	1/18	Van De Winkel et al. (1982)
Rat Islet Cells[a,c]	34.6	9/26	0	0/19	Huen et al. (1983)
RINm5F Cells[c]	33.3	6/18	0	0/14	Eskinazi et al. (1985)
HIT-T15 Cloned Hamster Beta Cells[b,c]	63.6	21/33	17.5	7/40	Grant et al. (1985)

[a] Cells fixed in Paraformaldehyde.

[b] Detected by Indirect Immunofluorescence.

[c] Detected by ^{125}I-protein A.

TABLE 6

Cytotoxic ICSA in Patients with IDDM and Control Subjects

Substrate	Patients with IDDM		Control Subjects		Reference
	%	Positive/ Tested	%	Positive/ Tested	
Hamster Islet Cells[c]	36.7	11/30	0	0/24	Rittenhouse et al. (1980)
Rat Islet Cells[b]	63.9	23/36	0	0/21	Dobersen et al. (1980a)
RINm5F Cells[b]	60.0	21/35	0	0/38	Kende et al. (1981)
RINm5F and RINm14F[a,b]	33.3	8/24	0	0/34	Eisenbarth et al. (1981a)
Mouse Islet Cells[c]	52.4	11/21	0	0/30	Sai et al. (1981)
RINm5F[d]	45.6	26/57	1.8	2/110	Toguchi et al. (1985)
RINm5F and RINm14B[a,d]	74.4	29/39	87.5	21/24	Cavender et al (1986)

[a] RINm14B cells synthesize somatostatin.

[b] ^{51}Cr-release assay.

[c] Trypan blue used to detect dead cells.

[d] Ethidium bromide used to detect dead cells.

concomitant presence of ICSA. In every study there are some individuals who have been recently diagnosed as having IDDM but whose sera are negative for ICSA. Of course these individuals may have exhibited ICSA prior to the manifestation of overt diabetes but as it is difficult to identify susceptible individuals this can be hard to monitor. This is one of the reasons the BB rat model is so important since it makes it possible to study the 'silent period' separating genetic predisposition and clinical onset of diabetes.

The other point to make about the data, especially in Table 5, is with regard to the presence of ICSA in control subjects. Although not common, ICSA is obviously present in a small percentage of normal healthy individuals with no family history of IDDM or autoimmune disease. The frequency of ICSA has been reported to range from 0-17.5% (excluding Cavender et al., 1986) in control subjects and therefore cannot be dismissed. Providing these figures are accurate they suggest that the presence of ICSA is not necessarily indicative of impending IDDM.

Dobersen and coworkers (1980a) demonstrated that 25% (14/56) of nondiabetic 1st degree relatives of diabetic probands exhibit cytotoxic ICSA. Moreover, Grant et al. (1985) found sera from 21% of nondiabetic age-matched paediatric patients were ICSA positive by indirect immunofluorescence. Ongoing studies are currently being undertaken which follow the ICSA positive relatives of probands with IDDM to see if they eventually become diabetic. Results from these studies will provide information on the accuracy of ICSA as signals or markers of IDDM. They may also help to clarify the role of these autoantibodies in IDDM.

The study by Cavender and coworkers (1986), referred to in Table 6, reported that the sera from 87.5% (21/24) control subjects exhibited complement-dependent cytotoxicity on the rat islet cell lines RINm5F and RIN14B. This number exceeds the 74% (29/39) of patients with IDDM, and there were no significant differences found in the titers between diabetic patients and control subjects. In preliminary studies using ^{51}Cr-labeled normal rat islets as target cells, these workers observed that control sera accounted for 20.5 $\pm$ 5.9% lysis whereas diabetic sera caused 37.1 $\pm$ 6.8% lysis with a marked overlap between the patient and control sera. Although there appears to be no corroborative evidence to support this startling discovery, the experiments in this study appear to be well controlled and these workers conclude that none of the assays describe to date adequately measure ICSA activity. This alarming accusation has not yet been resolved. It is possible that in the present study, as well as in other reports, ICSA were present but they were below the threshold of detectability. However, this would seem unlikely since many types of supposedly sensitive assays have been used and no patterns appear which correlate low positivity with a certain type of assay.

Yet another inconsistency in the literature concerns the claim that ICSA are specific to beta cells and do not cross react with other endocrine cells in the pancreatic islet. However Eisenbarth and coworkers (1981a) found that human diabetic sera mediated complement-dependent cytotoxicity against RIN14B cells as well as RINm5F cells. RIN14B cells are a cell line originating from the same tumor as RINm5F cells (Gazdar et al., 1980). They are predominantly somatostatin-producing cells and are therefore analogous to pancreatic

D cells. Dyrberg and coworkers (1984) have reported that BB sera was also cytotoxic to RIN14B cells. However, whereas somatostatin producing D cells often remain in islets from patients with IDDM (Orci et al., 1976; Gepts and Le Compte, 1981) immunocytochemical analysis of the diabetic BB rat pancreatic islets revealed pathological changes in D cells as well as B cells (Tannenbaum et al., 1981). The pathogenesis of diabetes in the BB rat and possibly in humans may therefore include other cells in addition to the B cells and may involve a variety of antigenic determinants. Furthermore, as previously mentioned, not only ICSA antibodies are detected in BB rats but also antibodies to thyroid, smooth muscle and gastric parietal cells, as well as anti-lymphocyte antibodies (Elder et al., 1982b; Like et al., 1982a; Like et al., 1982b; Dyrberg et al., 1982). However, neither the thyroid antibodies nor the parietal antibodies are accompanied by decreased function of the corresponding cells, even though lymphocytic infiltration of the thyroid was common in old BB rats (Wright et al., 1983). These observations suggest that the BB rat in general is affected by autoimmune reactions, but that the only clinical manifestation is diabetes.

In summary, the results from the in vivo study of cytotoxic ICSA in BB rats suggests that there is no correlation between ICSA and overt diabetes. One possible explanation for this lack of correlation could be because ICSA are of no importance in the pathogenesis of diabetes but appear as a consequence of beta cell destruction. Alternatively, the ^{51}Cr-release assay for cytotoxic ICSA may lack the sensitivity to measure the putative antibodies that are active in beta cell destruction, or perhaps the ICSA could encompass several islet cell

specificities and affinities. This would account for the large variation in the levels of ICSA within and between the rats. On the other hand, the beta-cell directed humoral immune response may very often be activated in diabetes-susceptible BB rats but additional factors then influence whether IDDM develops. Perhaps in some rats beta cell destruction may operate without ICSA whereas in others ICSA do contribute to the damage.

Whichever is the case, ICSA are clearly an interesting phenomenon and require further study. To this end, a monoclonal antibody (KT1) was produced which specifically lysed RINm5F cells and normal rat islet cells. By indirect immunofluorescence, this antibody also bound to cell surface antigens on both live and fixed cultured islet cells from Wistar rat neonates and RINm5F cells. Western blotting experiments revealed reaction to a 68-kD protein in RINm5F cell lysates. It is not clear if the 68-kD protein detected by KT1 is the same antigen as the human 64-kD islet cell antigen reported in the literature (Baekkeskov et al., 1984). Thus, Mab KT1 is unique in that it is the first monoclonal autoantibody to be produced which is cytotoxic to both untreated RINm5F and cultured islet cells. It, therefore, exhibits properties very similar to the islet cell surface autoantibodies found in the sera of BB rats. In future studies, Mabs such as this may play an active part in determining the role of autoantibodies in the destruction of pancreatic beta cells.

In contrast, a monoclonal autoantibody (IC_2), which binds to islet cells has been produced from a diabetic BB rat by Brogren and coworkers (1986), however, IC_2 was not cytotoxic to RINm5F cells or beta cells. Biochemical studies with IC_2 did not yield either a band in a Western

blot nor an immunoprecipitate. The reason for this could be that this Mab possibly recognizes a glycolipid rather than a protein (Shienvold et al., 1987). In addition, Uchigata and coworkers (1987) have recently reported the preparation of a cytotoxic monoclonal antibody (E5C2) from a diabetic BB rat which lyses rat islet cells only after they have been treated with neuraminidase. However, the target antigen recognized by E5C2 is unlikely to be a major target antigen in the immunopathological process that culminates in overt diabetes. The autoantigen(s) recognized by both the humoral and the cell-mediated autoimmune response appears to be present in untreated normal beta cells (Like et al., 1982b; Prud'homme et al., 1984). The evidence favours the underlying defect to be an abnormal immune regulatory system in the BB rat rather than an antigenically altered target tissue recognized by a normal immune surveillance system. Another antibody A1G12, produced following the fusion of spleen cells from a prediabetic BB rat and a mouse-myeloma cell line (P3X63), binds preferentially to islet cells of rat pancreas (Buse et al., 1983). This Mab appears to recognize an antigen on all types of islet cells and also gives an "anti-nuclear" rim fluorescence on all tissues studied, including pancreatic acinar cells. In contrast to the aforementioned rat monoclonal autoantibodies, the Mab KT1 may have clinical relevance as it exhibits properties more like the islet cell surface antibodies present in the sera of BB rats.

A monoclonal antibody to islet cell surface antigens has been produced from the hybridization of spleen lymphocytes from non-obese diabetic mice (Yokono et al., 1984). This monoclonal antibody was obtained from an autoimmune fusion involving nonimmunized diabetic

animals as in the present study. Mab 3A4 recognized a 64-kD component from hamster insulinoma cells, which is identical to the molecular weight cited by Baekkeskov et al. (1982 and 1984). However, 3A4 showed no complement-dependent cytotoxic activity, which was surprising since it belonged to the mouse IgG_1 class, which has binding capacity for complement.

A human monoclonal autoantibody has been produced by fusing circulating lymphocytes from a child with IDDM with a human myeloma cell line. This Mab, designated B6, binds preferentially to the A cells of frozen sections of human pancreatic islet, however, and will only bind to all islet cells on Bouin's fixed pancreatic sections. Therefore, B6 does not appear to resemble polyclonal ICSA in any way.

There are many reports available concerning monoclonal antibodies to islet cells produced by immunizing mice with islet cells or cultured insulinoma cells. However, Mabs produced in this fashion are generally heterospecific, reacting with a number of different types of tissues and cells (Eisenbarth et al, 1981b; Crump et al., 1982). One of these Mab (A1D2) produced following immunization with the rat islet cell tumor line RINm5F, is quite promising, however. It is specifically cytotoxic to rat islets in vitro and binds to a 24-kD glycoprotein. However it does not differentiate beta cells from other islet cell types (Kortz et al, 1982). Another murine heterospecific monoclonal antibody, HISL-17, was produced following immunization of mice with human islets and human insulinoma tissue. HISL-17 reacts with islets on frozen sections of human pancreas and is, therefore, likely to be an ICA-like antibody (Eisenbarth et al., 1984).

A different approach employed flow cyto-fluorometric-assisted screening to detect hybridomas producing monoclonal ICSA (Vissing et al., 1986). Mice were immunized with human islets prior to fusion of their spleen cells with mouse myeloma cells. Antibody-producing hybrids were cloned on the basis of their production of surface antibodies reactive with paraformaldehyde-fixed RINm5F cells by indirect immunofluorescence analysis in the fluorescence-activated cell sorter. A resulting Mab, 2G3, reacted with viable normal rat islets cells as well as RINm5F cells. However, none of the antigens detected by immunoblotting were entirely islet cell specific.

This summary of other monoclonal antibodies against islet cells is comprised of the most promising examples in the literature and there are many reports currently available of even less appropriate monoclonal antibodies. The inference is that the monoclonal antibodies obtained from autoimmune fusions are more specific to islet cells than those from immunization with xenogenic cells. Furthermore the present monoclonal antibody, KT1, appears to most closely fit the description of physiological ICSA in vivo. Unfortunately, the instability of the rat-mouse KT1 hybridoma has made it difficult to further characterize the antigen detected by KT1. Likewise it has not been possible to determine if this antigen is involved in the pathogenesis of diabetes in the BB rat. However, the presence of such autoreactive B cell clones in BB rats is of interest and deserves further study. Monoclonal antibodies, such as KT1, may be useful tools to clarify the role of ICSA and the relationship between the cellular and humoral autoimmunity in IDDM. Furthermore, even if ICSA are not the primary mediators of islet cell destruction, the autoantigens to which they

bind may be an important target antigen(s) involved in the immunopathogenetic process. Therefore, since the beta cell antigen(s) is central to the understanding of the pathogenesis of IDDM, monoclonal antibodies like KT1 should enable the identification and isolation of this autoantigen(s). Simple assay systems, such as an ELISA or RIA, could then be developed to detect beta cell antigen specific immune responses and active beta cell destruction. Since the chronic progressive beta cell dysfunction in IDDM can last more than 9 years in some individuals, this greatly extends the time period during which immunotherapy can be effective in preventing the development of diabetes. Thus, immunological markers to active beta cell destruction would be extremely beneficial for identifying potential insulin-dependent diabetics and for monitoring the effectiveness of immunotherapy (Wilkin and Armitage, 1986).

A. General Discussion and Conclusions

The pathological mechanisms involved in the induction of IDDM remain unclear. However, it appears from the results of the present study that there is a lack of correlation between the level of cytotoxic ICSA and overt diabetes in the BB rat. One reason for this could be that the beta cell directed humoral response might be activated in many of the BB rats but additional factors then influence whether or not IDDM develops. Also, there need not be a direct relationship between the antibody levels in serum and the state of the beta cell destruction in vivo. This still does not account for the presence of ICSA in such a large percentage of apparently healthy rats and in healthy 1st degree relatives of patients with IDDM. If indeed

ICSA was causing beta cell damage one would expect diabetes to occur in these ICSA positive individuals. However, this does not eliminate the possibility that ICSA are a contributing factor in the disease process and perhaps there is some crucial factor missing in these healthy individuals which protect them from becoming diabetic. Alternatively ICSA may be of no importance in the pathogenesis of IDDM, but may appear as a consequence of beta cell destruction.

The most likely pathogenesis for IDDM involves a combination of humoral and cell-mediated autoimmunity. The development of IDDM appears to be a multistep process in which formation of autoantibodies is often one of the steps. It is conceivable that the antibodies to pancreatic beta cells appear as a part of a broader immunological dysfunction associated with IDDM. Possibly autoimmunity develops spontaneously as a consequence of deterioration of immune control. The many immunological abnormalities detected in IDDM, may be due to an inherited defect of the immune system which participates in the autoimmune islet cell destruction. This might explain the spontaneous diabetes in the BB rat who becomes diabetic in gnotobiotic conditions and exhibit the full spectrum of physiological and morphological alterations observed in conventional diabetic BB rats (Rossini et al., 1979). However, a currently favored hypothesis for IDDM in humans is that a viral infection of islet cells or some other environmental agent may trigger an autoimmune response in genetically susceptible individuals. This also cannot be ruled out in the case of the BB rat since viruses can also be transferred vertically from the parents to the offspring. Another possible explanation is that insulin deficiency causes the immunological defects since it appears the immunoregulatory

abnormalities, such as islet cell antibodies, are transient and perhaps are corrected by insulin treatment. However, given the success of immunotherapy in preventing the disease in BB rats this theory is unlikely.

In conclusion, it is apparent that a direct relationship between the levels of ICSA in the serum and the state of beta cell destruction in vivo is unlikely to exist in BB rats. In addition the protective action of antilymphocytic serum (Like et al., 1979), neonatal bone marrow transfusions (Naji et al., 1981) and neonatal thymectomy (Like et al., 1982c) support a cell-mediated pathogenesis for diabetes in the BB rat. Transfusions of spleen cells have been shown to prevent spontaneous diabetes in susceptible BB/W rats, while T cell-depleted transfusions are ineffective (Rossini et al., 1984). Further evidence has come from studies on adoptive transfer of diabetes via the presumed effector cells. Diabetes can be transferred to Wistar Furth rats by ConA-activated splenocytes from acutely diabetic BB rats (Koevary et al., 1983b). On the other hand induction of diabetes in low incidence BB rats by ICSA positive diabetic sera has not been established.

Can the data obtained from the BB rat be applied to human IDDM? Although the BB rat model has many similarities to human IDDM there are a few important differences. For example, BB rats exhibit profound T cell lymphocytopenia which is necessary but not sufficient for the occurrence of diabetes. Human IDDM is not characterized by a severe T lymphocytopenia and therefore certain results of immunotherapy in the BB rat may not be applicable to the human disease. In the BB rat model, immune-modulation by lymphocyte transfusion seemed to have produced a lymphocyte chimera which reversed the lymphopenia and

prevented diabetes. This has as yet not been successful in human trials. However, human clinical trials using cyclosporin A were begun after successful use of the drug in the BB rat (Stiller et al., 1984).

B. Future Directions

There is still much work required to determine the role of islet cell surface antibodies in the pathogenesis of diabetes mellitus. To expand the results of the in vivo study, it would be advantageous to monitor insulitis and glucose intolerance as well as overt diabetes to determine if the incidence of ICSA correlates with the subclinical form of the disease in our BB rat colony. Furthermore, a selective breeding program of ICSA positive BB rats and ICSA negative BB rats should lead to a clearer understanding of the heritability of ICSA expression and enable analysis of the proposal that the expression of ICSA may be genetically independent from diabetes. It would also be interesting to test the possibility of gender related differences in the incidence of ICSA by studying a larger population of rats evenly divided for gender.

There are many interesting experiments which have yet to be carried out on the cytotoxic monoclonal antibody KT1. It is of great importance to show that the cytotoxic antibody is able to bind specifically to beta cells and not to other islet cell types. This would involve a double-label immunofluorescence assay on normal rat islet cells using a TRITC-anti-insulin antibody reagent in conjunction with the FITC-Mab KT1. If these reagents consistently bind to the same cells then this would indicate that KT1 is beta cell specific. Controls involving anti-somatostatin, anti-glucagon and anti-pancreatic polypeptide antibodies conjugated to TRITC would further support this

conclusion if these reagents were found not to bind to the same cells as those lit up by FITC-Mab KT1. It would also be advantageous to determine if KT1 binds or lyses RINm14B , the somatostatin producing cell line.

A different approach to determining if ICSA is important in the pathogenesis of IDDM would be to inject KT1 into prediabetic BB rat at an early age to see if this facilitates an earlier development of diabetes. This could also be tried in low incidence BB rats and normal Wistar Furth rats to see if KT1 could induce diabetes. This could potentially provide valuable information on the role of ICSA in diabetes. A more complete characterization of the autoantibodies detected by KT1 would be appropriate. Immunoprecipitation using KT1 on ^{35}S-methionine labelled RINm5F and rat islet cells lysates would provide additional information on the molecular weight of the antigen and since this method was used by Baekkeskov et al. (1984) it would help in determining whether KT1 detects the same 64-kD protein as the one detected by these workers. As discussed previously isolation of the autoantigen would be extremely beneficial and could lead to the development of assays for specifically reactive antibodies or possibly even T-cells in the blood from patients with IDDM.

Moreover, monoclonal antibodies are well suited to idiotypic analysis because of the relative ease of purifying the idiotype. Several studies in other animal models of autoimmune diseases and in human autoimmune disorders have demonstrated a high degree of cross-reactivity between idiotypes on the autoantibodies. Therefore a survey of cross-reactive idiotypes present in BB rats would be potentially very useful. The occurrence of cross-reactive idiotypes in

IDDM, especially those which are detectable by a monoclonal anti-idiotype reagent, may render feasible the possibility of regulating the production of pathogenic autoantibodies. This type of anti-idiotype therapy appears to have been affective in NOD mice using the Mab 3A4 (Yokono et al., 1986). They found that treatment of 6 week old NOD mice with antiserum raised against 3A4 prevented the occurrence and the progression of insulitis. At 8 weeks of age the levels of ICSA in the sera of these mice were significantly decreased.

It is clear that further work on ICSA is required. The existing knowledge of ICSA is incomplete with regard to antigen specificity and class of the antibody in vivo. ICSA of both IgM and IgG isotypes have been demonstrated in IDDM but it is not clear if the class switch from IgM to IgG production is related to the progression of the disease. Since IgG antibodies are more dependent on T cells than are IgM antibodies, the class switch could possibly signify a more active stage of the disease involving cell-mediated as well as humoral autoimmune processes. Therefore, it would be interesting to determine if there is an association between IgG as opposed to IgM antibodies and diabetes.

Once the autoantigen has been clearly identified, the next step would be to obtain this protein in the pure form. This could be accomplished by isolating the mRNA coding for this protein and cloning the corresponding cDNA. The protein could then be expressed in an appropriate vector in a bacterial host. Having available large quantities of the pure antigen would help to establish its role in the pathogenesis of IDDM. It is possible that the autoantigen(s) recognized by ICSA may also be a target for autoreactive T cells. If this is the case, purified antigen could be used to isolate these T

cells. These could then be cloned and their role in the pathogenesis of IDDM studied. Alternatively, anti-idiotypic antibody to ICSA may recognize these T cells and provide a means of eliminating them. There are many other directions for future research into the etiology of IDDM. Insulin-dependent diabetes in the BB rat and man is still not completely understood. Whether initial beta cell damage is accomplished by cell-mediated or humoral autoimmunity or both remains controversial. I have attempted to present some of the incongruity which exists within the current literature on IDDM. Part of this discordance can be attributed to the apparent heterogeneity within IDDM. The biases in patient selection and the limitations of current technology also contribute to the uncertainty about the exact nature of the immune defects in IDDM. Specific therapy for either the rat or the human will require the identification and isolation of the effector cell specifically responsible for the development of IDDM.

If this was achieved, production of monoclonal antibodies to deplete these effector cells would be one potentially useful method of immunotherapy. In addition, identification of the environmental agent(s) which may be responsible for initiating the autoimmune response, may lead to a prophylatic treatment against the disease.

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