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A STUDY OF
ANTI-PANCREATIC ISLET CELL ANTIBODIES
IN THE BB RAT

By

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Thesis submitted to the School of Graduate Studies
in partial fulfillment of the requirements for
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DEDICATION

This thesis is dedicated to my husband, Krishna and to my children, Anurag and Deepti, whose constant support and encouragement always kept me going. Without their constant support and encouragement, I could never have completed this work.

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I express my sincere thanks to Dr. I. Hynie and Dr. D.R. Pollard of the Laboratory Centre for Disease Control, Health and Welfare Canada, for encouragement, guidance and help throughout this study and allowing me to use the facilities at L.C.D.C. to carry out the work.

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SUMMARY

The work presented here is a summary of the study of anti-pancreatic islet cell surface and cytoplasmic antibodies in diabetes prone BB rats. About 40-70% rats of this colony develop insulin dependent diabetes mellitus spontaneously.

This study is the first (to the best of our knowledge) to demonstrate the presence of islet cell cytoplasmic antibodies (ICA) in the BB rat serum. We have demonstrated the presence of ICA by the indirect immunofluorescence method using Bouin's fixed rat pancreatic sections as substrate.

Binding levels of circulating ICA in the BB rat were quantified by measuring the immunofluorescent intensity of the islet cells incubated with rat sera. The immunofluorescent intensity was measured by a photodetector attached to the Zeiss microscope. The signals from the photodetector were sent to the Zonax computer and were read using the software 'BIOSCAN PROGRAM'. This enabled us to eliminate the subjective scoring of immunofluorescent intensity on the slides.

Islet cell cytoplasmic antibodies (ICA) were present in diabetic animals as well as in potential diabetics who never developed clinical symptoms. Sera from control rats did not show any immunofluorescence specific to the islet cells.

A two step indirect ^{125}I labelled protein A binding method was developed to measure the levels of islet cell surface antibodies (ICSA) in sera from BB rats. In agreement with our earlier observations (Pollard et al.,1983), this method

confirmed the presence of ICSA in the circulation well before the appearance of clinical symptoms.

Islet cell surface antibodies could not be removed from the serum by absorbing the sample with an equal volume of packed rat spleen cells. Antibodies responsible for complement dependent cytotoxicity to the islet cells could not be verified in-vitro, in the serum samples studied.

A prospective study of ICA and ICSA was carried out by measuring the levels of both types of antibodies in weekly samples drawn from twenty BB rats (10 males and 10 females), starting well before the earliest onset of the overt symptoms (age 6-7 weeks) and carried through for 30 weeks. Nine of the twenty animals, 7 females amd 2 males, became overtly diabetic during the course of the study.

Both ICA and ICSA were found in all the animals, whether clinically diabetic or healthy. It appears that a syndrome of abnormal immune state always precedes the disease, but that this state may not necessarily lead to clinical diabetes in all the animals. Considerable fluctuations were observed in the binding levels of the two antibodies. In a few instances the antibodies, more often ICA than ICSA, were undetectable in one week's specimen but reappeared in samples from subsequent weeks.

No significant correlation was observed between the time of onset of overt clinical symptoms and the pattern of ICA or ICSA levels.

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B Cells	Insulin producing Beta cells present in islets of Langerhans
BSA	Bovine serum albumin
DM	Diabetes Mellitus
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
HBSS	Hanks balanced salt solution
HLA	Human leukocyte antigens
ICA	Islet cell cytoplasmic antibodies
ICSA	Islet cell surface antibodies
IDDM	Insulin-dependent diabetes mellitus
MHC	Major histocompatibility complex
NIDDM	Non-insulin-dependent diabetes mellitus
OHA	Oral hypoglycemic agents
PBS	Phosphate buffer saline
RITC	Rhodamine isothiocyanate
RT1	Rat histocompatibility complex
SD	Standard Deviation

INTRODUCTION

Over many years the understanding of diabetes mellitus gradually evolved from a concept of one disease with many different manifestations to a concept of several etiologically unrelated diseases which share glucose intolerance as a common manifestation. The term "juvenile onset" diabetes was replaced by "insulin dependent" or "Type I" dibetes mellitus (IDDM) in recogintion of the accumulated evidence that it is not the age of onset but the distinct etiology and pathogenesis which sets this rare condition apart from the more prevalent but less serious "non-insulin-dependent or "Type II" diabetes mellitus.

Further studies documented the autoimmune component of the pathogenesis of IDDM, the role of certain genotypes in the susceptibility to this condition and the unique pathological changes in the islets of Langerhans of victims of the disease. However, to this date the etiology of IDDM is uncertain and knowledge of the pathogenesis is only superficial. Until recently one of the reasons of our inability to have a comprehensive understanding of the disease was the lack of a suitable animal model which would allow in-vivo expetiments closely mimicking human IDDM. The arrival of the diabetes prone BB rat proved to be a nature's boon to scientists. In the recent past, experiments done on the BB rat have provided some invaluable information about human IDDM.

The BB rat was discovered in 1974 in a commercial colony of Wistar derived rats at Bio-Breeding Laboratories, Ottawa

Canada. About 40-70% of the BB rats maintained as a random bred colony at Health and Welfare Canada develop diabetes spontaneously with a number of clinical, pathological and immunological analogies to human IDDM.

In the present work we have utilized these rats to study the antibodies to pancreatic islet cells which are important immunological markers of human IDDM. Two types of islet cell antibodies are associated with the disease; one type is directed towards the cell surface antigen (ICSA) and the other towards the cytoplasmic determinants of islet (ICA) cells. The significance of islet cell antibodies in the disease process is not known, the main reason being the lack of standardization of methods to determine these antibodies.

ICSA have been demonstrated in the BB rat also, however the previous attempts to demonstrate ICA have not been successful. In this work we aimed to establish the presence of ICA in BB rats. Secondly we have tried to develop methods to quantify the levels of both types of antibodies and employ these methods to study the prevalence of these antibodies in relation to the presence and absence of diabetes, the time of onset of the disease and the age of the animals. As the BB rat originally appeared in this city, we were able to use the animals from the original non-diabetic Wistar colony as controls.

The significance of these results is discussed and a salient review of the literature is presented.

LITERATURE REVIEW

DIABETES

Diabetes Mellitus (DM) is not a single disease but a complex group of disorders that share carbohydrate intolerance in common. Besides carbohydrate metabolism, lipid and protein metabolism is also affected by the disorder. DM is characterized by hyperglycemia, polyuria, polydipdsia, polyphagia glycosuria and fatigue. Hyperglycemia is the consequence of a total lack, deficiency or improper utilization of insulin. It leads to chronic complications of DM such as neuropathy, retinopathy, myocardial infarction or gangerene of the lower extremeties. DM and its complications are the third leading cause of death and the second leading cause of blindness in Western society (Garber et al., 1983). The estimated prevalence of DM is approximately 2% of the population in North America.

A. Classification Of Diabetes Mellitus

In 1979 the National Diabetes Data Group (NDDG) classified the disorder into various groups on the basis of familial, metabolic and immunological considerations (table 1). Two groups, classified as insulin-dependent diabetes mellitus or Type I and non-insulin-dependent diabetes mellitus or Type II, account for the great majority (>90%) of patients with diabetes mellitus. The main characteristics that distinguish insulin-dependent diabetes (IDDM) from non-insulin-dependent diabetes (NIDDM) are summarized in table 1.

IDDM is characterized by the appearance of clinical

TABLE 1

CLASSIFICATION OF DIABETES

(a) Insulin-Dependent (Type I)	(b) Non-Insulin-Dependent (Type II)
Clinical Features	
Usually growth onset	Usually maturity onset
Always insulin dependent	Often controlled by diet
Abrupt onset	Gradual onset
Ketosis prone	Usually not Ketotis prone
Islet cell antibodies present	Absent
Association with HLA	No association with HLA
Pathology	
B cell mass < 10%	Moderate reduction
Insulitis at onset present	Absent

(c) Gestational Diabetes - Glucose intolerance during pregnancy

(d) Impaired Glucose Tolerance - When blood glucose is intermediate between normal and those considered diabetic.

(e) Secondary Diabetes - Hyperglycemia resulting from:

a) Destruction of beta cells - pancreatitis, hemochromatosis surgical extirpation, streptozotocin (glucosamine antibiotic cytotoxic for β cell)

b) Excess of "diabetogenic hormones" - acromegaly, Cushing's, pheochromocytoma, glucagon tumours.

c) CNS disorders - trauma or encephalitis

d) Defective insulin receptor

symptoms accompanied by severe insulin deficiency. The insulinopenia resulting from near total disappearance of the insulin producing B cells renders the affected individuals dependent on exogenous insulin for their survival. Insulin therapy prevents death from diabetic ketoacidosis but it does not prevent late complications of the disease. The appearance of IDDM at onset is highly variable. In the majority of cases, the onset of the disease is abrupt, though, in some patients polydypsia and polyuria may be present for a month or even longer before the appearance of acute ketosis.

IDDM is associated with certain histocompatibility antigens, autoimmune diseases, circulating anti-pancreatic islet cell antibodies and insulitis. Formerly it had been termed as juvenile-onset, because the majority of the indviduals affected by the disorder are young persons, but it can occur at any age.

Patients with non-insulin dependent diabetes (NIDDM) may have normal, depressed, or even elevated insulin levels. Therefore, they are not dependent on exogenous insulin as their life support and are relatively resistant to ketosis. They may develop ketosis under certain circumstances such as stress or infection, and may need insulin to correct symptomatic or persistant hyperglycemia. Affected individuals are normally obese, but they can also be lean. In obese NIDDM, obesity is suspected as one of the etiological factors and glucose balance in these individuals can be improved simply by diet and weight loss.

The present study is aimed at developing methods to measure the levels of anti-pancreatic islet cell antibodies prevalent in insulin dependent diabetes mellitus (IDDM) and utilizing these methods to study the significance of these antibodies in the pathogenesis of the disease. Therefore, the forthcoming review will concentrate on IDDM.

Since insulin deficiency plays a key role in the development of IDDM, and insulin is produced by the B cells found in pancreatic islets of Langerhans, a brief description of islets of Langerhans is presented before reviewing IDDM.

B. Islets of Langerhans

Islets of Langerhans represent a complex mixture of endocrine cells. They are distributed throughout the endocrine pancreas and make up an average of 1-2% of total volume of pancreatic tissue (Hellerstrom, 1977). The islets are composed of endocrine cells, blood vessels, nerve fibres and connective tissue. At least four cell types, A, B, D and pp are present in the pancreatic islets of all mammalian species.

Two distinct populations of islets have been observed in the mammalian pancreas by immunocytochemical techniques (Baetens et al.,1979). The islets from the body and tail of the pancreas, also known as dorsal area, have been characterized by the presence of glucagon producing A cells along the periphery with very few, if any, pp cells. In contrast, the islets from the lower part of the head of pancreas (also called ventral region) contain pp cells in the periphery but very few glucagon containing A cells. B and D

cells are uniformly distributed in the islets from both locations in man as well as in rats. Insulin producing B cells form the central core of the islets.

Endocrine Cell Types

The insulin producing B cell is the predominant cell type in the islets of Langerhans. In humans these comprise about 60% (Hellerstrom, 1977) and in rats about 70 to 80% (Baetens et al.,1979) of the total number of islet cells. There are very few cell types in the human body which are functionally better characterized than the B cell.

The A cells are the second best defined islet cells. These are the source of pancreatic glucagon and make up to about 25% of the human endocrine pancreas. In the rat, mouse and hamster, these are typically located in the islet periphery; whereas, in man they occur both on the islet periphery and along the islet capillaries (Hellerstrom, 1977).

The D cells are the source of pancreatic somatostatin or a compound that has a somatostatin-like immunoreactivity (Polak et al.,1975). While D cells usually comprise less than 10% of the islets of laboratory animals, they seem to be relatively more abundant in human islets. They are located immediately inside the periphery of the islet, making close contact with the A and B cells.

The fourth and the most recently discovered islet cell type is known as the pp cell (Larsson et al.,1976). As the name implies, these cells are the source of pancreatic polypeptide.

II. INSULIN-DEPENDENT DIABETES MELLITUS

"IDDM as it is recognized clinically, most probably represents several similar conditions having differing causes and pathogenetic mechanism" (Craighead, 1978). The failure of insulin producing B cell function seems to be the single most important pathogenic feature.

A. Epidemiology Of IDDM

Insulin dependent diabetes mellitus accounts for 15% of all human diabetics (Rossini, 1983a). Epidemiological studies show that the incidence of the disease varies from 7.7 to 30 per 100,000 population per year (Zimmet et al.,1982). The higest incidence has been reported in Finland and relatively low incidence is reported in the Chinese, Polynesians, Japenese, Indians, American Indians, Eskimos and South African blacks. Most studies indicate equal occurrence of the disease in both sexes (Cahill et al., 1981), but a few studies indicate a slight preponderance of males in younger diabetics (Creutzfelt et al.,1976; Nerup et al.,1980; Laporte et al.,1981) and about 29% female preponderance in diabetics above the age of 31 years (Cudworth et al.,1982).

The onset of IDDM seems to follow a seasonal pattern, with increased incidence of new cases in autumn and late winter followed by a lower incidence rate in summer (Craighead et al., 1978; Gamble, 1976). An inversion of above pattern has been reported in Southern Hemisphere (Durruty et al.,1977). Children who develop IDDM in their preschool years do not show any seasonal pattern (Gamble et al.,1976; MacMillan et

al.,1977). Recent evidence has indicated that diabetes associated with the HLA DR3 haplotypes shows significant variation with season; whereas, that associated with HLA DR4 haplotypes does not (Weinberg et al.,1984).

B. Islet Cell Morphology In IDDM

Morphological studies done on the pancreas of insulin dependent diabetics soon after their death reveal two distinct pathological conditions in the islets: a marked decrease in the number of the B cells and lymphocytic infiltration of the islets. Pancreata of young diabetics, who died almost immediately after the diagnosis of the disease, show a loss of about 90% of insulin-producing B cells, while a nearly complete disappearance of B cell mass has been observed in the pancreas of patients who died a few months to years after the onset of the disease (Gepts, 1965). Histological studies indicate that B cells do try to regenerate, but the attempt at B cell regeneration is apparently curtailed by an inflammatory reaction that destroys these cells shortly after their appearance. A few B cells can still be detected in about 50% of the diabetic subjects who have had the disease for less than 10 years and in 18% of those with longer duration of the disease. (Gepts et al.,1981).

After the destruction of B cells, the pancreata are left with a large number of pseudo-atrophic islets composed of well preserved A and D cells (Orci,et al., 1976 and Gept et al., 1978). In a few cases, hyperactive islets have been observed, although they are much rarer than the pseudo-atrophic islets

(Gept et al., 1957; Maclean et al.,1959). These are irregular in size and contain normal A and D cells but large and degranulated B cells. Gepts et al.(1978) also observed a marked proliferation of pp cells. Islets composed of pp cells only are derived from the epithelium of medium size ducts.

Lymphocytic infiltration of the islets, referred to as insulitis, is invariably observed in the pancreas of acute Type I diabetics dying within two months of diagnosis. This lesion is specific for insulin-dependent diabetes. Insulitis is detected more often in hyperactive, B cell-containing islets, but remnants of lymphocytic infiltrations are still found in some pseudo-atrophic islets (Gepts, 1965). The cause of the B cell destruction in IDDM has not been elucidated. All morphological evidence suggest the implication of both genetic and environmental factors in the etiology of IDDM.

C. Etiology of IDDM

i. Genetic Factors: Familial Occurrence and Association with HLA Antigens

IDDM tends to be familial though heritable influences are not as strong in the transmission of IDDM as they are in NIDDM. Studies have shown (Walker et al., 1980) that its occurrence in siblings is 1:20, a 25-fold greater risk than that for the general population. IDDM may be more common in offsprings of diabetic fathers than those of diabetic mothers (Warram et al.,1984)and the youngest child in the family appears to be more susceptible to the disease (Flood et al.,1982). Studies done on identical twins (Barnett et al.,

1981) demonstrate that 50% of the twins studied are concordant for IDDM. Individuals carrying certain haplotypes for polymorphic HLA antigens appear to have inherited the increased genetic predisposition for the disease, suggesting that inheritance is linked to one or more genes of chromosome six, situated in proximity to the HLA loci. HLA -A, -B, -C and DR antigens belong to the major histocompatibility complex (MHC), located on the short arm of the sixth chromosome in man. MHC contains genes coding for cell surface proteins involved in a variety of immunological phenomena, including allograft rejection, initiation of antibody formation and cell-cell interaction.

Pooled studies (Cahill et al., 1981) done on families with more than one person affected indicate that on the average 58% of the siblings with IDDM share the same HLA haplotype, 37% share at least one parental HLA haplotype, and only 5% do not share any parental haploidentity in contrast to the expected random distribution of 25:50:25. A significant increase in the frequency of certain HLA antigens, B8, B15, B18, Cw3, Cw7, D/DR3 and/or D/DR4, and a reduced frequency of antigens B7 and D/DR2 has been observed in IDDM individuals (Singhal et al., 1973;Nerup et al., 1974 and Cudworth et al., 1975). A positive association of BfF1 with IDDM has also been reported (Kirk et al., 1979 and Rich et al., 1984). BfF1 is a rare allele of properdin B(Bf), a component of the alternative pathway of complement, whose genetic locus lies between HLA-B and HLA-DR. The primary positive association between chromosome six

markers and IDDM susceptibility, however, appears to occur with HLA antigens D/DR3 and D/DR4; and, the primary negative association with the antigen D/DR2. About 95% of diabetic probands have been found to carry antigen D/DR3, D/DR4 or both and DR3/DR4 heterozygotes carry more risk than either DR3 and DR4 homozygotes (Nerup et al., 1982; Rich et al., 1984).

There are many individual antigens that tend to show a strong positive association with IDDM. Enough data have been gathered from large population studies on ethnic Caucasians to show that individuals carrying haplotypes D/DR3-B8-Cw7, D/DR3-BfF1-B18-Cw5, D/DR4-B15-cw3 and D/DR4-B40-Cw3 have a high relative risk for developing the disease; while the haplotype D/DR2-B7 seems to offer a strong protection against development of IDDM (Christy et al.,1979; Cudworth, 1982; Rich et al., 1984). It has been suggested that the reduction in DR2 frequency in IDDM could be secondary to the primary increase in DR3 and DR4; or, it could be that DR2 is in linkage disequilibrium with a putative immune response gene near or in the HLA-D region which prevents the B cell damage.

Most IDDM patients (85-90%) do not have affected first degree relatives. A population study done on both familial and sporadic cases of IDDM indicates that the familial cases of IDDM have stronger association with DR3/DR4 antigens than the sporadic cases (Christy et al., 1979). Results of the 8th International Tissue Typing Workshop indicate that in African blacks and Japanese the HLA antigens D/DR8, instead of D/DR3 and/or D/DR4, confer the susceptibility to IDDM. Thus in all

populations two HLA-DR antigens seem to confer susceptibility to the disease.

HLA association with IDDM brings it in close relation to some of the endocrine and nonendocrine diseases that have been reported to have a positive association with HLA DR3 and in which autoimmune and/or viral mechanisms may be pathogenic (Schernthaner et al., 1982). The nature of the mechanism by which these genes in the HLA chromosome region control the susceptibility to insulin-dependent diabetes mellitus is not yet known. The initiation of the disease in genetically predisposed individuals may be caused by some enviromental insult such as viral infection or chemical injury to the insulin producing B cells. It is apparent that heritable factors play a part in some cases but it is not certain whether or not genetic influences are critical in all the cases. The genetic factors may be determinant of the cell's susceptibility to injurious agents.

ii. Enviromental Agents in the Etiology of IDDM

The association of IDDM and viral infections has been recognized for many years (Maugh et al., 1975). Certain B cell cytotropic strains are thought to be involved in the initiation of the disease in humans as well as in laboratory animals, but the degree of the causative role of viral agents in the pathogenesis of IDDM is highly debatable. At one end of the spectrum there are cases in which viruses seem to be directly responsible for the B cell destruction. At the other end there is a large number of cases, with no direct evidence

of viral etiology.

A number of studies indicate a temporal relationship between IDDM and antecedent viral infection (Craighead, 1978; Notkins,1977). The seasonal variation in the incidence of IDDM correlates with the incidence of Coxsackie virus infection (Gamble, 1969a). Higher titers of antibodies against the Coxsackie B-4 virus are observed in newly diagnosed IDDM patients than in normal subjects or IDDM subjects of greater than three months duration (Gamble,1969b). The presence of inflammatory cells in the pancreatic islets of some newly diagnosed IDDM cases has been mentioned above (Gepts, 1957). All these observations are suggestive of the variable degree of the causative and provocative role played by a virus in the initiation of the disease. Epidemiologiacal observations suggest that IDDM follows Coxsackie virus infection by weeks (Gamble et al., 1969b), mumps infection by months to several years (Sultz et al., 1975) and intra-uterine exposure to rubella virus by a number of years (Menser et al., 1978).

There are reported cases where viruses seem to be directly responsible for the development of IDDM but the number of such cases is extremly low. One such case is that of a 5 year old girl who suffered severe myocarditis and IDDM 19 days after operative suture of an atrial septum defect (Gladisch et al., 1976). Three other cases of acute ketotic diabetes following myocarditis of suspected viral etiology have been reported (Gibbs, 1974) . In another case (Yoon et al., 1979) the Coxsackie B4 virus was successfully isolated from a culture of

pancreatic homogenate from a boy who developed fatal IDDM after influenza like infection. The genetically susceptible mice innoculated with the Coxsackie B4 virus isolated from the above case developed IDDM, resulting in hyperglycemia and B cell damage. Further documentation provided by this particular case included a rise in viral antibody titre both in the patient and the infected mice, and the finding of the viral antigen in the cells of the patient's brain. What role autoimmunity played in this case is not known, since islet cell antibodies were not determined. However, cases of severe viral infection leading directly to destruction of B cells are rare in the experience of most diabetetologists.

Genetically susceptible mice have also been reported to develop IDDM after being infected with mumps, Coxsackie B3 and reovirus type 1 and type 3 (Onodera et al., 1981). Mice infected with reovirus type 1 presented a complete picture of classical IDDM including polyendocrinopathies and antibodies to islet cells. Antibodies to anterior pituitary hormone, growth hormone and insulin were also detected in these infected mice (Onodera et al.,1981, Haspel et al.,1983). It is suggested that autoantibodies to various endocrine target tissues can be induced by injecting neonatal animals with rheoviruses (Haspel et al., 1983).

In some patients after mumps infection, the presence of transient islet cell cytoplasmic antibodies have been reported without any apparent effect on glucoregulatory system (Helmke et al., 1980). In another study, viral cytopathology was

observed in the panoreata of only 10% of 250 children with fatal infections caused by various viruses, and, B cell destruction and inflammatory infiltrates were found in the islets of 4 out of 7 cases with Coxsackie B virus (Jenson et al., 1980). Moreover, if the children had survived, the degree of observed B cell damage, induced by various infections, was not sufficient to cause diabetes.

Antibodies to Coxsackie virus are found in half of the population without apparent effect in the individuals. Similarly there is a high prevalence of mumps in the general population, but not everyone who has had mumps infection becomes diabetic. The clinical, epidemiological and pathological observations suggest that external enviromental agents like viruses may play a role of variable importance in the initiation or provocation of the disease in some genetically susceptible individuals. However, there is no conclusive demonstration that IDDM is always due to a viral infection.

The role of certain chemicals has also been implied in the destruction of B cells. Six cases of acquired IDDM after the ingestion of rodenticide (Vecor) have been reported (Karam et al., 1980). Immunohistochemical studies done on the panoreatic tissues obtained from the two deceased cases demonstrated extensive damage to the B cell, while A, D and pp cells appeared normal. Circulating islet cell antibodies were detected in two out of the four surviving cases. There was no association with known susceptible HLA haplotypes.

III. PATHOGENESIS OF IDDM

Autoimmunity appears to be the most important factor in the pathogenesis of insulin dependent diabetes mellitus. For the past few years implication of immune mechanisms, both cellular and humoral, has been hypothesized in its pathogenesis. Evidence has accumulated in support of the hypothesis: the observation of insulitis in the newly diagnosed IDDM individuals (Gepts et al.,1981; Bottazzo,1984); the prevalence of circulating islet cell cytoplasmic (Lendrum et al., 1976; Bottazzo et al., 1974) and/or surface (Lernmark et al.,1978; Doberson et al., 1980) antibodies in the sera of 80 to 95% IDDM subjects; the prevalence of islet cell antibodies in some organ specific autoimmune diseases (Irvine et al., 1977a); the characteristic association of IDDM with certain HLA antigens; the observation of high frequency of D/DR3 antigens in IDDM and other organ specific autoimmune diseases (Svejgaard et al. 1980); the raised levels of low affinity E-rosette forming cells (which are believed to represent most of the killer or K cell population) in approximately 50% of diabetics (Pozzilli et al., 1979); the increase in numbers of Ia-positive T cells (Jackson et al., 1982); the increased complement-mediated as well as antibody dependent cellular cytotoxicity in the sera of IDDM patients (Charles et al., 1983).

A. Cell Mediated Immunity In IDDM

The main evidence for cell mediated immunity in IDDM comes from histopathological studies done on the pancreatic tissue

from newly diagnosed diabetics. When first observed, (Weichselbaum et al.,1910) insulitis (lymphocytic infiltration of the pancreatic islets) was considered a rare condition. In later studies insulitis was observed in the pancreas of 50% (Junker et al., 1977) to 66% (Gepts, 1965) of IDDM patients dying within a few months to a year of diagnosis. It is observed more often in young subjects, but has also been reported in patients as old as 60-70 yr (Lecompte et al., 1972). In most cases of insulitis only a few islets are affected. It is not found in the individual with a duration of disease longer than a year, and it may consist of only a few infiltrating cells (Gepts, 1981) and, therefore, can escape detection (Doniach et al., 1973).

Infiltrating cells surrounding individual islet or groups of pancreatic islets have recently been identified on frozen pancreatic sections as mononuclear cells (Bottazzo, 1984). Cytotoxic/suppressor T cells seem to constitute the bulk of the cellular infiltrate; but some T helper and, to a lesser extent, natural killer cells have been detected. Most interestingly, plasma cells have been observed emanating from pancreatic vessels. IgG molecules have also been observed, both within the islet cells and coating their surface (Bottazzo, 1984).

Lymphocytes from diabetics have been found to be toxic to cultured human insulinoma cells (Huang et al., 1976; Maclaren et al., 1975) and also have been shown to inhibit insulin release by rat pancreatic islets in-vitro (Boitard et al.,

1980). In-vitro inhibition of insulin secretion from cultured mouse pancreatic islet cells is observed in the presence of lymphocytes from 92.5% of the diabetics studied (Boitard et al., 1984), suggesting the involvement of lymphocytes in the destructive process of B cells. A decrease in concanavalin A-inducible suppressor cell activity has been detected only in the early state of the disease (Buschard et al., 1980), but according to another report (Lederman et al., 1981) the decrease in suppressor cell activity persists even late in the course of the disease. Fairchild et al. (1982) have reported antigen-specific (islet cell antigen) hypofunction of T cell activity in the majority of IDDM patients. However, abnormal suppressor T cell function has also been reported in non-diabetics who are DR3 and DR4 positive, suggesting that the abnormality in the immune mechanism may be related to genetically linked factors (Ambinder et al., 1981; Jaworski et al., 1983).

The number of B lymphocytes in IDDM is usually normal but increased levels have been observed when islet cell cytoplasmic antibodies are present (Bersani et al., 1981). Furthermore, the proportion of IgG producing B cells is reported to be higher than normal in the individuals who are positive for islet cell cytoplasmic antibodies.

Studies done on peripheral T lymphocytes give equivocal results. According to one report utilizing monoclonal Okt3 antibodies, in the majority of diabetic cases the number of T cells is lower than that observed in normal controls (Galluzzo

et al., 1984). However, others (Pozzilli et al., 1983; Buschard et al., 1983) report that the total number of peripheral T lymphocytes remains unchanged in the disorder. Decreased levels of high affinity E rosette forming cells (Cattaneo et al., 1975) and increased levels of low affinity E rosette forming cells have been reported in the early course of the disease (Pozzilli et al.,1979). The low affinity rosette forming cells are believed to contain most of the killer (K) cells (West et al., 1978). In most of the cases, the levels of K-cells have been reported to come down to normal levels during the course of the disease, except in cases with persistent ICA (Pozzilli et al., 1979).

In contrast to the above report, when the proportion of natural killer cells was measured using monoclonal antibodies Leu-7 and Leu-11a, most of the IDDM subjects had slightly lower levels of these cells than the controls; but in NIDDM subjects the levels of natural killer cells were found to be significantly elevated (Herald et al., 1984). Furthermore, in some of the newly diagnosed IDDM subjects the ratios of helper/inducer and suppressor/cytotoxic T cells were found to be elevated (Horita et al., 1982; Galluzzo et al., 1984). On the other hand 80% (17/21) long standing diabetics and 63% (15/23) newly diagnosed diabetics had a normal ratio for these cell types (Galluzzo et al., 1984).

The observed discrepancies in the results of lymphocyte subset population studies could be due to the pathogenetic heterogeneity of the disease (Craighead et al.,1978) or it

could also be due either to the use of different methods or to different monoclonal antibodies. The observed wide variety of abnormalities in the proportion of peripheral blood lymphocyte subsets points towards the involvment of the cellular immune mechanism in the pathogenesis of IDDM.

B. Humoral Immunity in IDDM

Over the past ten years a number of investigators have reported that sera from patients with IDDM contain antibodies that react with pancreatic islet cells. An increase in the levels of circulating immune complexes have also been reported (Irvine et al.,1978; Di Mario et al.,1980) in newly diagnosed IDDM individuals. At least two types of circulating antibodies to pancreatic islet cells have been demonstrated in the sera of many individuals affected with IDDM. One of these types reacts with the determinants present in the cytoplasm of islet cells (Lendrum et al., 1975; Bottazzo et al.,1974; Delprete et al., 1977; Dobersen et al.,1979) and the other type is specific to the islet cell surface (Maclaren et al.,1975; Lernmark et al.,1978; Dobersen et al.,1980; Pujol-borrel et al.,1982). Islet cell antibodies, both cytoplasmic and cell surface, have been found to be organ specific for the endocrine pancreas, but do not appear to be species specific, as these have been shown to react with islet cells from the normal pancreas of man (Lendrum et al.,1975), rat, mouse (Lernmark et al., 1981) and monkey (Krell et al.,1984). Islet cell antibodies have been shown to belong to the IgG class (Lendrum et al.,1976).

1. Islet Cell Cytoplasmic Antibodies

Historically islet cell cytoplasmic antibodies (ICA) were first demonstrated by Bottazzo et al (1974) in some of the patients showing evidence of organ specific autoimmunity, both diabetics and non-diabetics. Sera from these patients contained multiple antibodies to endocrine glands (Bottazzo et al.,1974; MacCuish et al., 1974) including antibodies to pituitary (Bottazzo et al.,1975), indicating the possibility of an autoimmune process underlying the lesion. Subsequently ICA were demonstrated in the sera of newly diagnosed young IDDM patients (18 years or less at the time of diagnosis). The follow up studies on these patients revealed that ICA prevalence is maximum at the time of diagnosis. The prevalence rate appears to decrease with time, it drops to 45% after one year and to 25% after two years of the diagnosis (Lendrum et al.,1976). In some of the older patients ICA seem to persist for a long time, sometimes for a life time. Most of the cases with persistent antibodies are associated with some other polyendocrine diseases.

Crossectional studies done on the diabetic subjects show that ICA are present in 60 - 85% of newly diagnosed IDDM cases (Lendrum et al.,1976; Irvine et al al.,1977a; Mustonen et al.,1984). ICA are also found in 4.7 - 6% of non diabetics with organ specific autoimmune diseases, in 3 - 3.8% of first degree relatives of IDDM patients and in 0.5% of the normal poulation (Lendrum et al;1975;Irvine et al.,1977a; Bottazzo et al.,1978).

Cytoplasmic ICA are reported to react with all the endocrine islet cell types (Bottazzo et al.,1978). These authors have suggested that either there is a common antigen present in all the cell types or there are multiple antibodies reacting with different antigens present in the various cell types. It is of interest that ICA did not show any reaction with the gastric glucagon and somatostatin producing cells, indicating that the human antibodies are able to identify subtle differences in the structure of cells in different organs (Bottazzo et al.,1978).

The disappearance and reappearance of ICA is observed in the sera of some unaffected first degree relatives and about 27% of affected IDDM subjects. (Spencer et al.,1984). In some cases ICA have reappeared after 5 years of negative results. The intermittent appearance of ICA has led to the suggestion that fluctuations in ICA may reflect silent intermittent infection by insulotropic viruses (Bottazzo, 1984). Disappearance of ICA in some unaffected first degree relatives is explained by suggesting that some indivduals who are genetically susceptible to the disease may possess a natural protective mechanism against ongoing autoimmune damage. The disappearance of antibodies in these cases may be related to remission of islet cell destruction.

Some reports indicate that ICA may appear in circulation long before the disease becomes manifest (Irvine et al.,1977a; Gorush et al.,1981; Srikanta et al.,1984). A number of studies have indicated that many ICA positive cases have proceeded to

develop insulin-dependent diabetes. In one study, (Gleishman et al., 1984) 18 out of 67 patients, all susceptible to IDDM development, treated with oral hypoglycaemic agent (OHA) were found to be positive for islet cell antibodies and 71% (14/18) of these required insulin within 3.7 years of OHA treatment. In another study of OHA treated cases 23 out of 160 were ICA positive and 61% (14/23) of these required insulin within three years (Irvine et al.,1980a).

The presence of ICA has also been reported in 10% (5/50) of pregnant women with impaired glucose tolerance, and 3 out of these 5 ICA positive women later developed IDDM (Steel et al.,1980)

In an ongoing prospective study of subjects at high risk for IDDM, 75% (24/32) have been found to be positive for ICA and 10 out of the 24 have so far become diabetic (Srikanta et al.,1984). This study includes first degree relatives of diabetic probands (including monozygotic twins) and subjects with transient glycosuria. In two of the monozygotic twins the chemical symptoms of the disease appeared more than 7 years after the detection of ICA. These same authors (Srikanta et al., 1985) have recently reported observing a low insulin response to intravenous glucose challange in six out of 12 ICA positive first degree relatives of IDDM probands, indicating that a decrease in B cell functional reserve is correlated to the presence of ICA.

It seems that an early appearance of ICA, as described in above cases, may be an indicator of ongoing Beta cell

destruction.

ii. Islet Cell Surface Antibodies

The second type of antibodies found in the sera of a large number of diabetic patients is directed towards the membrane of pancreatic islet cells (Lernmark et al.,1978). Studies done on newly diagnosed diabetics demonstrated the presence of ICSA in the sera of 30 to 87% of IDDM cases using insulin producing cell lines or pancreatic islet cells from various sources as substrate (Maclaren et al.,1975; Lernmark et al., 1978; Van de Winkle et al.,1982; Pujol-Borrel et al.1982). ICSA have also been reported in 4 out of 9 non-diabetics with glucose intolerance (Lernmark et al, 1978), in 5 out of 11 NIDDM subjects (Van de Winkle et al.,1982), in two out of 10 long standing diabetics with coexistent autoimmune diseases (Pujol-Borrel et al.,1982), and in 5.5% normal healthy children (Lernmark et al.,1978; Van de Winkle et al.,1982).

ICSA, like ICA, are evanescent (Freedman et al.,1979). In one study, ICSA were found in 41% of patients with the disease of less than one month duration, decreasing to 15% after 1-12 months and to 12% after 1-5 years (Freedman et al., 1979). However the prevalence of ICSA increased to 29% in the patients with a duration of the disease over 5 years, and most of these patients were found to be negative for ICA. One possible explanation for the increased prevalence rate of ICSA could be the higher mean age (45 yr) of the subjects with longer duration of the disease as compared to the mean age (21 yr) of those with the disease duration of less than one year

(Freedman et al.,1979). Islet cell surface antibodies and cytoplasmic antibodies do not always correlate with each other and they may occur independently of each other (Freedman et al,1979; Pujol-Borrel et al.,1982), suggesting that two different antigen-antibody systems may be involved.

ICSA are specific to the islet cells as these antibodies cannot be absorbed out of the sera with spleen or blood cells nor with the acetone powder of liver (Van de Winkle et al., 1982). The observation that ICSA are specifically directed towards the B cells (Van de Winkle et al., 1982) suggests their involvement in an autoimmune destruction of panoreatic islets leading to the preferential lysis of the B cells.

Sera from a large number of diabetic patients are capable of mediating a complement-dependent cytotoxic reaction in-vitro against pancreatic islets cells from hamster (Rittenhouse et al.,1980), rat (Dobersen et al., 1980) and man (as qouted in Kanatsuna et al.,1982). However, the presence of cytotoxic antibodies in the sera does not necessarily imply that these are diabetogenic since their presence has been reported in 25% of first degree relatives of diabetic probands. These may not have any cytotoxic activity in-vivo. Diabetic serum containing ICSA has been shown to inhibit glucose-induced insulin release from the perifused islet cells in the presence of complement (Kanatsuna et al,1982). It has been suggested that the increased levels of Natural Killer (NK) cells in IDDM patients (see above) may attack islet cells after interacting with the antibodies bound to cell surface.

Further evidence for autoimmunity comes from the ability of diabetic sera, containinig ICA and/or ICSA, to immunoprecipitate an antigen of 64k molecular weight from the islets of non diabetic cadaver donors (Baekkeskov et al.,1982). In addition to the 64K antigen, diabetic sera were able to immunoprecipitate another antigen having a molecular weight of 38K from the islets obtained from an HLA DR3 positive donor. The unique antigen expressed in the islets of HLA DR3 positive individuals may be one of the factors leading to the precipitation of the disease under special circumstances, such as viral infection, in these individuals.

An increase in circulating immune complexes has been reported in a large number of IDDM cases, however, the antigens involved in these complexes and their role in IDDM are still unknown (Irvine et al.,1978; Di Mario et al., 1980). In most cases AgAB complexes tend to disappear within a few months after diagnosis, with a decline that parallels the ICA decline (Pozzilli et al.,1982), but stay high in individuals with persistent ICA. Ag-Ab complexes consisting of islet cell antigen and islet cell antibodies may be the consequence of islet cell damage caused by external agents. These complexes may play a pathogenic role in the development of the disease by activating the natural killer cells and rendering them specifically cytotoxic to islet cells (Irvine et al.,1980b).

iii. Methods used for Detecting Islet Cell Antibodies

ICA and ICSA have been detected by a number of methods using pancreatic islets in different forms and from various sources.

ICA are generally determined by the standard indirect immunofluorescence method, using fresh frozen sections of human pancreas from blood group 'O' cadaveric donors. The pancreata from blood group 'O'donors are required to eliminate the non specific exocrine reaction from donors of other groups. Recently ICA in human serum have been demonstrated using pancreata obtained from monkeys (Krell et al.,1984). Besides fresh frozen pancreas, Bouin's fixed pancreatic sections have also been used to demonstrate ICA (Dobersen et al.,1979; Kolb et al., 1983). The reliability of the method has been challanged (Rosenbloom, 1983) on grounds of non-specific binding of the serum to exocrine cells. However, when a set of samples was tested on unfixed sections and Bouin's fixed sections from the same pancreas, the results were not significantly different than those obtained on two different O group pancreata (Kolb et al:1983). Bound ICA can also be detected by immunohistochemical procedure using glucose oxidase (Krell et al.,1984).

Recently a method has been developed in our laboratory (Pollard, D.R. and Mancino, L. unpublished results) to partially quantify ICA by measuring the immunofluorescent intensity of islets. This method has been used for the results presented here.

The presence of ICA in IDDM patients has also been demonstrated by using the abilities of the antibodies to fix complement. The complement fixing ICA (CF-ICA) were found in the sera of 70% of newly diagnosed diabetics (Bottazzo et

al.,1980). CF-ICA appear in the circulation later than the conventional ICA and are the first to disappear after onset of the disease. It has been suggested that CF-ICA belong to a separate sub-species of islet cell antibodies, and, may be a more selective marker of ongoing B cell destruction (Bottazzo et al.,1980). However the complement fixing ability of the antibodies in the course of the disease appears to depend on the titre of conventional ICA (Bruining et al.,1984; Gleichmann et al.,1984). All the sera, positive for conventional ICA with a titre of at least eight, were found to be positive for CF-ICA also.

ICSA can be detected by an indirect immunofluorescence method using insulin producing cells or pancreatic islet cells from various sources as substrate. ICSA were demonstrated in 87% of sera using insulinoma cells (Maclaren et al,1975), in 30-60% of patients using rat or ob/ob mouse pancreatic islet cells (Lernmark et al.,1978; Van de Winkle et al.,1982) and in 81% of cases using cultured cells from human fetal pancreas (Pujo-Borrel et al.1982). Islet cell surface antibodies raised in rabbits against rat and ob/ob mouse pancraetic islets have been partially quantified using a radio-ligand assay method (Lernmark et al.,1980). A large variation in the prevalence of ICSA, in newly diagnosed patients, reported by different studies is probably due to the use of a variety of substrates and a lack of standardization of the tests. Even two pancreata from human blood group 'O' did not give exactly same results (Kolb et al.,1983).

IV. ANIMAL MODEL OF IDDM: THE "BB" RAT

Though there are numerous animal models of diabetes, especially mice models, the majority is characterized by hyperglycemia secondary to hyperphagia and insulin resistance. These models serve as examples of NIDDM and not IDDM. The Chinese hamster can acquire an insulinopenic ketosis type of diabetes (Gerristen et al.,1967) but not cellular or humoral autoimmunity. However the BB rat provides a good animal model in which pathogenesis similar to human IDDM can be mimicked.

A. The BB Rat

The diabetes prone BB rat was first discovered in an outbred Wistar colony at Bio-Breeding Laboratories, Ottawa, Ontario, Canada, (Nakhooda et al., 1977). The BB rat develops IDDM spontaneously with clinical, pathological and immunological features anlaogous to those of human IDDM. (Nakhooda et al., 1978; Seemayor et al., 1983;Marliss et al., 1982). The acute onset of the disease is characterized by severe hyperglycaemia, glycosuria, hyperglucagonemia, ketoacidosis, weight loss and reduced concentration of circulating and pancreatic insulin. Without insulin treatment most of the ketotic animals will die within 1-2 weeks of the overt onset of diabetes (Nakhooda et al., 1978). In different colonies of BB rats maintained at various places, the prevalence of diabetes varies from 40-70%. It can vary from 10-80% by further selective breeding of the animals (Yale et al.,1984). The peak incidence of the disease occurs between the age of

60-40 days and both sexes are equally affected (Nakhooda et el., 1978).

The histopathological findings in the pancreas of these rats are similar to those described in the early stages of human IDDM: decreased insulin content, degranulation and necrosis of the B cells in the islets (Seemayor et al.,,1983) and insulitis of varying intensity in the early phase of the disease. A mild form of insulitis has been observed in some young normoglycemic animals, presumably prediabetics (Tannenbaum et al., 1981), and in all lymphocytopenic rats with or without glucoregulatory abnormalities (Poussier et al., 1982). An intense insulitis is accompanied by the selective destruction of the B cells. The B cells are completely destroyed and disappear within 7-21 days after glycosuria (Nakhoda et al., 1978). This is accompanied by a decrease in the number of A, B and pp cells (Marliss et al.,1982). Electron microscopic and immuno-histochemical studies have revealed that infiltrating cells include macrophages, T and B lymphocytes.

A number of studies have reported profound T cell lymphopenia in both peripheral blood and lymphiod tissues of the BB rat (Jackson et al.,1983; Elder et al., 1983). The most affected lymphocytes appeared to be the T helper cells which are reduced to one third of the normal value. Diabetes is always accompanied by lymphocytopenia, though lymphocytopenia has been observed without the disease (Marliss et al., 1982). Other characteristics of the syndrome reported

to this date are; lymphocytic thyroiditis but not abnormal thyroid function (Sternthal et al., 1981), islet cell surface antibodies (Pollard et al., 1983; Dyrberg et al.,1984) and antibodies to gastric parietal cells, smooth muscles and thyroid colloid (Like et al., 1982a; Maclaren et al., 1983; Elder et al., 1982). The BB rat may, threfore, have an abnormal immune response, which predisposes it to the development of IDDM.

The presence of circulating ICSA have been reported in most of the animals from BB rat colonies with high prevalence of IDDM, and in a proportionately smaller number of animals from the colony with low prevalence of the disease (Dyrberg et al.,1983). The antibodies are present long before the onset of the disease (Pollard et al.,1983; Dyrberg et al., 1984).

Genetic studies done by crossing BB rats with other strains (Colle et al.,1983; Jackson et al.,1983b) indicate that at least a u haplotype of RT1 region derived from the BB rat is essential for development of the disease (RT1 is the major histocompatibility complex of rat).

Two theories are being postulated for the development of diabetes in the BB rat: a two genes theory and a one gene theory. According to the former (Colle et al.,1983; Jackson et al.,1984) two genes are required for the development of the disease in the rat: (i) a gene responsible for the expression of u haplotype or in close linkage with it, and (ii) another gene which is responsible for T cell lymphopenia, and is not linked to RT1 locus. The second, A single gene or a gene

cluster theory with incomplete penetrance is advocated by Butler et al., (1983). The gene or the gene cluster required for the development of the disease appears to be linked to the gene responsible for u haplotype at RT1 locus.

B. Theraputic Trials

The strong evidence pointing towards autoimmunity as one of the contributory factors in the development of IDDM in man and the BB rat led to trials of immunosupressive therapies in an attempt to reverse or prevent the acute syndrome of IDDM. In the BB rat the administration of anti-rat lymphocyte serum normalized the plasma glucose levels in 30% of acutely diabetic rats and prevented overt hyperglycemia in non diabetic litter mates (Like et al 1979). The prevalence of the disease in susceptible rats was reduced from 27 to 3 percent after complete thymectomy and from 59 to 7 percent after total lymphoid irradiation (Like at al., 1982b; Rossini et al., 1984). Administration of Cyclosporin A, immunosuppressive drug, to weanling animals prevented the disease in all the treated animals. Within forty nine days after the termination of the therapy 25% of the animals became diabetic (Laupcis et al., 1983). Transfusion of blood from a non diabetic subline of the rats prevented the development of the disease in all the susoeptible BB rats, however, insulitis was observed in one of the eighteen transfused animals (Rossini et al.1983b).

C. Similarities and Differences of IDDM in Man and The BB Rat

In addition to a similar clinical symptomatology the features of the disease common to both man and the BB rat are insulitis, circulating islet cell surface antibodies and a tendency to show other organ specific autoimmunity. However lymphocytopenia is not observed in man and the attempts made to demonstrate islet cell cytoplasmic antibodies in the rat have been unsuccessful to this date (Like et al., 1982b; Maclaren et al., 1983; Elder et al.,1982). The presence of circulating anti-lymphocyte antibodies has also been reported in BB rats and these have been shown to have a close correlation with the ICSA (Dyrberg et al.,1983, 1984).

The most striking difference is observed in the etiology of the disease in the two species. While human IDDM is a sporadic condition, rarely affecting first degree relatives of diabetics, and is probably caused by the combination of genetic and environmental factors, the disease in the BB rat is familial with very high prevalence and without any evidence of the role of environmental factors in the etiology. Despite the differences between human IDDM and the disease in BB rats the number of similarities between the two makes the BB rat a valuable model for further exploring.

STATEMENT OF THE PROBLEM

Although human IDDM and the diabetes in the BB rat may be two etiologically different diseases, the clinical and biochemical manifestations are almost identical and immune abnormalities appear to be similar. Therefore the BB rat provides an execellent opportunity to explore the sequence of pathological changes and biochemical abnormalities preceding the onset of clinical symptoms of diabetes, which may contribute towards our understanding of pathogenesis of human IDDM. It is not possible to do a similar systematic study in humans prior to the on set of IDDM. However an observed pattern of abnormalities in the BB rat can be compared with some random observations in humans to postulate a possible analogy.

The presence of antibodies to pancreatic islet cell surface (ICSA) and cytoplasmic determinants (ICA) is an immunological abnormality associated with human IDDM. There are a few reports of finding islet cell antibodies in first degree relatives of diabetics and subsequent development of the disease in some of these previously healthy individuals. In our earlier study we have shown that in the BB rat ICSA precede the onset of the disease. The presence of ICA have not been demonstrated in the rat so far. The significance of either type of antibodies is not known. A systematic study of these antibodies in the BB rat, starting from well before the onset of the disease, can help in understanding the disease process.

Our hypothesis is that an autoimmune condition is inherited, which may not always result in the clinical manifestation. The clinical symptoms might be expressed only if more than 90% of the beta cells in the pancreatic islets are destroyed. However all the affected animals including those clinically asymptomatic, may possess some detectable marker such as circulating islet cell antibodies. The hypothesis is based on the observation that inheritance of diabetes in the BB rat does not show any mendelian pattern even in the colonies with high prevalence of the disease, suggesting the occurrence of a single gene mutation.

The present work was undertaken with the following objectives in mind:

(i) to see if antibodies to cytoplasmic determinants of pancreatic islets (ICA) are present in the BB rat.

(ii) to develop methods for the quantification of ICA and ICSA levels.

(iii) to follow the appearance of ICA/ICSA in the individual animals by drawing samples of blood weekly, starting well before the onset of the disease and following through the life time of these animals.

(iv) To investigate if there is any correlation between the levels of ICA/ICSA and the time of onset of the disease.

MATERIALS AND METHODS

I. MATERIALS

A. ANIMALS

Test sera came from the spontaneously diabetic BB Wistar rat which is maintained by the Health Protection Branch as a random bred closed colony.

Control sera came from the rats derived from the original non diabetic parent Wistar colony.

The animals are kept in air conditioned room (20°C, 50% relative humidity) with twelve hours of light and twelve hours of darkness. They are fed Purina Lab Chow 5001 and water ad libitum.

In BB rats urinary glucose levels are determined twice a week and an animal is classified 'diabetic' on the basis of glycosuria. The diabetic animals are kept on PZI insulin administered daily. The prevalence of diabetes in the colony is approximately 40-70% and the average age of onset is about 85 days.

B. Anti-Sera

Rabbit anti-rat IgG, guinea-pig anti-bovine insulin antibodies, Fluoresoein isothiocyanate (FITC) conjugated anti-guinea-pig IgG, and rabbit anti-rat IgG Rhodamine isothiocyanate (RITC) and goat anti-rabbit RITC conjugates were all bought from Miles Laboratories Inc., Elkhart, USA. FITC labelled rabbit anti-rat IgG was purchased from General Diagnostics, Canada. Goat anti-guinea-pig gamma globulin and

rabbit anti-glucagon antibodies came from Calbiochem Behring Corp. Lajolla, USA. Affinity purified goat anti-rat IgG $F(ab')_2$ fragment was bought from Bio/Can Scientific Inc. Mississauga, Canada.

C. Radiolabelled Chemicals

Insulin RIA kit and ^{125}Iodine labelled Protein A were bought from Amersham, Mississauga, Canada. ^{51}Cr (Sodium Chromate) was purchased from New England Nuclear, USA, and ^{125}Iodine (Sodium Iodide) from Atomic Energy, Canada.

D. Tissue Culture Products

Tissue culture Media 199, Hanks balance salt solution (HBSS) powder, Swim's medium 77, fetal calf serum (FCS), penicillin and streptomycin were all were purchased from GIBCO Laboratories, Grand Island U.S.A. Falcon 15x60 mm tissue culture dishes and Dynatech 96 wells 'removawell' microtitre plates were bought from Fisher Scientific Limited.

E. Chemicals and Other Products

Rat insulin standard was a gift from Dr. N. Begin-Heick, Department of Biochemistry, University of Ottawa, Ottawa, Canada.

Complement- Rabbit complement, Low-Tox-H and Low-Tox-M, were bought from Cederlane Laboratories, Hornby, Canada.

Ficoll-400, Protein A and Sephadex G-50 medium were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Bovine serum albumin (BSA) fraction IV RIA grade, HEPES and EGTA [Ethyleneglycol-bis-(B-amino-ethyl-ether)N,N'-Tetraacetic acid] were bought from Sigma Chemicals. Trypan Blue came from

NCS diagnostics, Mississauga, Canada, and Hypaque sodium was bought from Winthrope, Aurora, Canada.

All the chemicals used were of reagent grade.

F. Preparation of Media and reagents.

Standard Medium- Medium M199 supplemented with penicillin 20 units/L, Streptomycin 200 mg/L, 10% FCS and 5.5 mM glucose was used as standard culture medium.

Swim'sM-Swim'sM was prepared with Swim's medium 77 containing 1% BSA, 20 mM HEPES, .02% sodium bicarbonate, and 1 mM EGTA, and, the final pH was adjusted to 7.3.

Hanks Buffer-Hanks balanced salt solution was supplemented with 2% fetal calf serum and 20 mM HEPES.

Phosphate Buffer Saline (PBS)- It consisted of 8.5 g sodium chloride, 1.07 g disodium phosphate (anhydrous) and 0.39 g monosodium phosphate ($2H_2O$) to 1 litre with distilled water and pH 7.1.

Dulbecco's BSA-I- Dulbecco's BSA-I contained 0.5% BSA in standard Dulbecco's buffer.

Dulbecco's BSA-II Dulbecco's buffer was supplemented with 0.9% BSA and 0.09% sodium azide after adjusting the pH to 7.2

Ficoll solution- Ficoll solution contained 11.6% Ficoll 400, 19.0% Hypaque sodium and 0.7% HBSS in double distilled water.

Trypsin solution- Swim'sM containing 0.2% Trypsin and 0.015 M DNase was used for cell suspension.

Trypan blue- Four parts of 0.5% of Trypan blue was mixed with one part of five times normal saline just before using

for cell viability test.

Glassware- All the tubes and pasteur pipettes used for isolation of islets and making cell suspension were siliconized with 1% prosil solution.

II. METHODS

A. Collection and Storage of Sera

All test and control animals were bled from jugular vein and the whole blood was left to clot overnight at 4°C. Next day the sera were recovered by centrifugation of the clotted blood in a clinical centrifuge and stored at -40°C in 200ul aliquots until used for determination of islet cell antibodies. Approximately 1.5 ml of blood was drawn each time from the animals used for sequential bleedings and 5-7 ml was drawn from the control animals used for one time bleeding.

B. Immunofluorescence on pancreatic sections

Freshly removed pancreata from a 3-5 days old control rats were fixed in Bouin's solution and embedded in paraffin wax using the standard method. Pancreatic sections, 7 microns in thickness, were mounted on pre-cleaned microscope slides coated with albumin fixative, and were heat dried overnight. After deparaffinizing the sections, approximately 75 µl of test sera (diluted 1:4) were applied to the sections and the section were incubated for 3 hr in a moist chamber at 37° The slides were washed three times, 10 min each, in 0.01 M PBS. After air drying, approximately 100µl of fluorochrome labelled anti-rat IgG (dilution 1:100) was applied to the sections. The slides were incubated for 1.5 hr and washed

again three times in 0.01 M buffer. In order to stain A or B cells, the sections were incubated with anti-glucagon or anti-insulin antibodies followed by incubation with rhodamine labelled anti-rabbit IgG for A cells and fluorescein labelled anti-guinea-pig IgG for B cells. Air dried sections were permanently mounted with Eukit and scored under Zeiss photomicroscope equipped with two different epifluorescence Zeiss filter combinations: the filter combination no. 10 consisting of exciter BP 450-490, and barrier BP 520-561 for FITC and the combination no. 14 consisting of exciter BP 510-560 and barrier LP 590 for RITC.

C. Measurement of Immunofluorescent Intensity

All the slides incubated with test sera were read objectively with a photodetector (a photomultiplier tube Hamamatsu R929) mounted on photometer head of the Zeiss microscope and interfaced with the Zonax computer. The values of immunofluorescent intensities were measured against a Zeiss fluorescent standard (47-27-56) using a software package 'Bioscan Program'.

Before scoring a set of slides the instrument was always calibrated with the fluorescent standard (henceforth referred to as reference standard). The intensity of the standard was set as 100% and the intensity with window closed was set at 0%. In this arrangement the photomultiplier's response is linear up to 140% intensity of the standard. All the readings on the slides were measured as percentages of the standard's intensity.

D.Isolation and Culturing of Islets

Pancreata were aseptically removed from 5 to 7 days old neonatal rats from our control colony. The islets of Langerhans were isolated from pancreata under sterile conditions by the method of Pollard, et al., (1983) using collagenase digestion and Ficoll-Hypaque gradient. The isolated islets were maintained in 60x15 mm culture dishes under standard culture conditions until they were used for any test. In order to maintain the islets in long term cultures, old medium was carefully aspirated from the culture dishes and was replaced with fresh medium every third or fourth day. Fibroblast growth in the long term cultures was prevented by feeding the cultures with the medium supplemented with 0.28 mg% Thimersol (Etylmercurithiosalicylic acid sodium salt).

E.Insulin RIA

In order to determine the viability of islets the levels of insulin released by the islets maintained in the cultures was measured. The media aspirated from the culture plates were centrifuged in a clinical centrifuge and the supernatants were stored in 200 µl aliquots until the day of insulin determination. The insulin levels were determined using Amersham Insulin radioimmunoassay (RIA) kit.

F. Radio Labelling of Protein A and IgG

Protein A and anti-rat IgG were labelled with ^{125}Iodine by the method of Greenwood and Hunter (1962). Twenty five µg of the each protein was labelled with 500 µci of ^{125}I (Sodium

Iodide). Reaction was carried out for 2.5 min in the presence of 12 µg chloramine-T and terminated with 25 µg of sodium-meta-bisulfate. The labelled proteins were separated from free iodide by passing through a 9x150 mm Sephadex G-50 column.

G. Islet-cell Suspension

Medium was aspirated from 8-10 plates containing well adhered islets and the islets in the plates were rinsed with 5 ml of Swim'sM. The islets were then dislodged by incubating each plate for 2-4 min with 1 ml of 0.02% trypsin solution at 37°C, transferred to 16x125 mm tubes and dispersed by shaking the tubes for 2 min at 37°C on a wrist action Burrel shaker The suspension was immediately washed twice with cold Swim'sM for 5 min at 800 rpm and the pellet was resuspended in 1 ml of standard medium containing 15% FCS. The cell count and the viability was checked by haemocytometer using the Trypan blue exclusion test. After diluting the suspension to 15 ml with the medium the cells were allowed to recover under standard culture conditions for at least 90 min before being used for any test. In order to culture the cells in microplates the pellet was suspended in the standard medium supplemented with 0.01 mM 3-isobutyl-1-methylXanthine, cell density was adjusted to approximately $2.5x10^5$/ml and 200 µl of the suspension was dispensed in each well of microtitre plates.

H. Testing of islet cell suspension for the Presence of A and B cells

Islet cells cultured in microtitre plates for at least 48 hr were washed with PBS buffer at room temprature, using

200 µl of the buffer for each well. The cells were fixed in Bouin's solution for 30 min, and washed twice with 70% alcohol for 30 min, and finally rinsed with PBS. The cells were then incubated in duplicate for 90 min with 60 µl of each: anti-insulin antibodies (dil. 1:20), anti-glucagon antibodies and PBS. The anti-sera and PBS were removed from plates by inverting the plate on a Kimwipe and the cells were washed three times with PBS (leaving the buffer in the wells for five min each time). After removing the buffer, 60 µl of Iodinated protein A (approximately 50,000 cpm) was added to each well and incubated for 45 min at 37°C. The wells were washed three times with the buffer, separated, and each well was transferred to 12x75 mm tubes to be counted in LKB Ultrogamma counter.

I. Absorption of sera with spleen cells

Cell suspensions were prepared from spleens as described by Ford (1979). Donor animals came from the control colony. Equal volumes of the test sera and packed spleen cells were incubated for 45 min at 25°C, shaking gently. The absorbed sera were centrifuged at 5000g for 4 min and supernatants were stored at -40°C until tested.

J. One step radio-ligand assay in tubes

The density of the dispersed islet cells was adjusted to $1x10^6$/ml and aliquots of 60 µl each were dispensed into 12x7 mm culture tubes. An equal volume of test sera diluted (1:2) with Hank's buffer were added to the tubes containing cells and the tubes were incubated for 35 min at 37°C shaking gently

in a shaking bath. After washing twice with Hank's buffer at 1000 rpm for 5 min, the pellet was resuspended in 50 µl of iodinated protein A or anti-rat IgG and incubated for another 40 min. The cells were washed twice again with Hank's buffer and the pellets were counted in LKB Ultrogamma for 2 min each.

K. Two step radio-ligand assay in microtitre plates

The islet cells cultured in microtitre plates for at least 48 hr were washed with Dulbecco's BSA-I. Test sera, 60 µl each (diluted 1:4 in Dulbecco's BSA-I), were added to the wells in duplicate and incubated for 90 min at 37°C. The sera were removed by inverting the plate on a Kimwipe and the cells were washed three times with Dulbecco's BSA-I leaving the buffer in wells for 5 min each time. The cells in each well were then incubated for 45 min with 60 µl of anti-rat IgG diluted 1:50, followed by three washes in buffer. Finally, the cells were incubated with 60 µl of ^{125}I-protein A (approximately 50,000 cpm) for further 45 min, washed three times and each well was counted for two minutes in the gamma counter.

L. Two step radio-ligand assay in tubes

Media was removed from the cell suspension (made by method II) by centrifugation at 1100 rpm for 3 min and the pellet was resuspended in 1 ml of cold Dulbecco's BSA-II. After adjusting the cell density to $1x10^6$/ml, aliquots of 75 µl cell suspension and 25 µl test sera in duplicate were incubated at 8°C for 90 min in a shaking bath. After washing the cells twice with 5 ml of cold Dulbecco's BSA-II, (1000 rpm, 5 min) the pellets were resuspended in 60 µl anti-rat IgG (dil.1:50),

and, incubated for 50 min. The cells were washed three times. Approximately 60,000 cpm of ^{125}I labelled protein A in a volume of 50 µl were added to each pellet and incubated for 40 min. The cells were washed three times and the pellets were counted for two minutes each in the LKB gamma counter.

M. Cytotoxicity Assay using Chromium-51 release method

Approximately 80,000 cpm of sodium ^{51}chromate were added to islet cell cultures in microtitre plates and incubated for three hours at 37°C under standard culture conditions. After removing the free radioisotope by gentle aspiration the wells were washed twice with Dulbecco's BSA-I (200 µl per well). 50 µl of the buffer was added to four wells and 50 µl of heat inactivated test sera were added in triplicate to the remaining wells and the plate was incubated for 35 min at 37°C. After washing the wells once with Dulbecco's BSA-I, 200 µl of the buffer was added to two of the four wells incubated with the buffer and to one of the three wells previously incubated with the different test sera, 200 µl of rabbit complement diluted 1:16 was added to the remaining wells and the plate was incubated further for 35 min. An aliquot of 190 µl was transferred from each well to 10x75 mm culture tubes and counted in LKB Ultrogamma counter. One hundred percent chromium release was determined by lysing the cells in two wells (incubated with Dulbecc's BSA-I) with 200 µl of 0.05% Triton X-100.

N. Statistical Analysis and Nomenclature

In all the tables presenting the results, BB denotes the

spontaneously diabetic BB Wistar rat and numbers on the left are serial numbers assigned to the animals.

P values have been calculated using 'Wilcoxen rank sum' test also known as 'Mann-Whitney U' test.

The graphs have been plotted with sharp plotter using software package 'Micro-Key'.

RESULTS AND DISCUSSION

I. ISLET CELL CYTOPLASMIC ANTIBODIES

A. Delineation of Topography of Islets

Topography of islets in Bouin's fixed pancreatic sections was delineated by staining glucagon producing A cells with anti-glucgon antibodies and insulin producing B cells with anti-insulin antibodies using the indirect immunofluorescence method. Pancreatic sections incubated with rabbit anti-glucagon antibodies were stained with anti-rabbit IgG rhodamine isothiocyanate (RITC) conjugate and the sections incubated with guinea-pig anti-insulin antibodies were stained with anti-guinea-pig IgG fluorescein isothiocyanate conjugate. Anti-glucagon antibodies produced diffused immunofluorescence in A cells located along the periphery of the islets and anti-insulin antibodies showed a diffused cytoplasmic fluorescence in the large central portion of the islets, corresponding to the B cells. Photographs of stained islets are shown in fig.1a and 1b. These observations helped to identify the islets in pancreatic sections under phase contrast microscopy and presented a topographical picture of rat islets.

B. Demonstration of Islet Cell Cytoplasmic Antibodies (ICA)

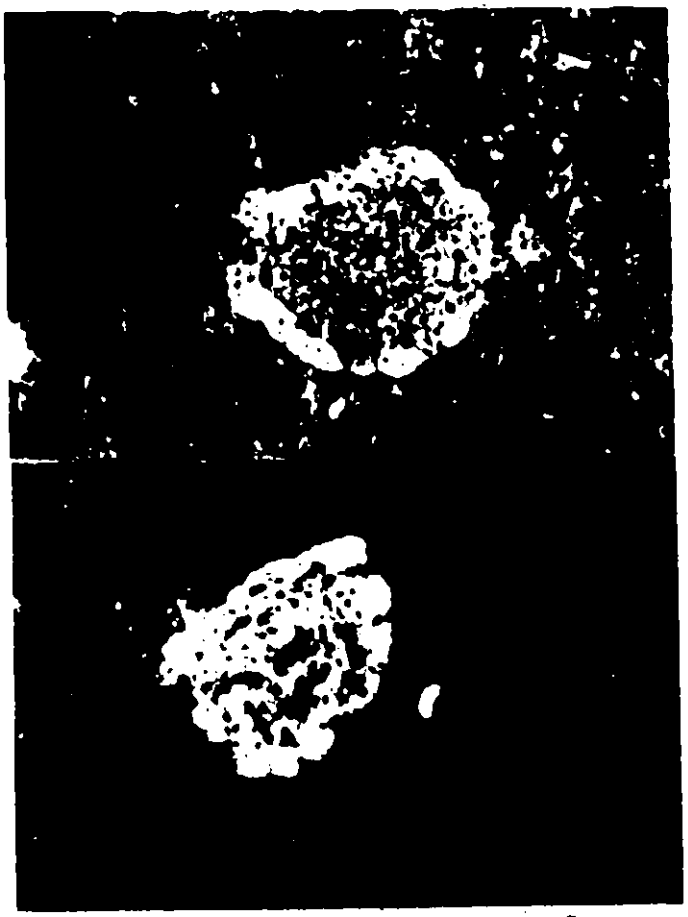
Islet cell cytoplasmic antibodies (ICA) were demonstrated in BB rat sera by the indirect immmunofluoresence method using Bouin's fixed pancreatic sections as substrate. The sections were incubated with BB rat sera or control rat sera, followed by incubation with FITC anti-rat IgG conjugate. BB rat sera

FIGURE 1

TOPOGRAPHY OF RAT PANCREATIC ISLET

(a)

(b)



Rat pancreatic sections were sequentially incubated with (a) rabbit anti-glucagon antibodies and goat anti-rabbit IgG RITC conjugate; (b) guinea-pig anti-insulin antibodies and rabbit anti-guinea-pig IgG FITC conjugate; (a) fluorescine glucagon producing A cells along the periphery of the islet; (b) fluorescing insulin producing B cells forming the centre core of the islet.

showed a bright immunofluorescence specific to the cytoplasma of the islet cells, while the exocrine cells on the section were found to be much darker than the islets (fig. 2a.). No such immunofluorescence was observed in the sections incubated with control serum (fig. 2c). Phase contrast photomicrographs of the sections incubated with BB rat serum and control serum are shown in fig. 2b and 2d respectively.

C. Specificity of the Reaction

Islet cell specific immunofluorescent staining obtained with BB rat sera could be blocked by incubating the sections with an excess of unlabelled anti-rat IgG prior to incubation with FITC anti-rat IgG.

After incubating the sections with serum samples (1:4 dilution in buffer), they were incubated with various concentrations of unconjugated anti-rat IgG and finally with FITC rabbit anti-rat IgG. A strong positive immunofluorescence specific to the islet cells was observed with diabetic BB rat sera but not with control sera in the absence of unconjugated anti-rat IgG. The immunofluorescence was partially blocked on the sections incubated with 25-50 µg/ml of unlabelled anti-rat IgG and was completely blocked when anti-rat IgG concentrations exceeded 50 µg/ml (Table 2). These observations indicate that (i) binding of rat sera to islet cell cytoplasmic determinants has the character of antibody-antigen reaction, (ii) rat ICA are probably immunoglobulin G.

Subsequently the sections were incubated simultaneously with a diabetic rat serum and guinea-pig anti-insulin

FIGURE 2

DEMONSTRATION OF ICA

Pancreatic sections were incubated (a) with diabetic rat serum and (b) with control rat serum, followed by the incubation with rabbit anti-rat IgG. FITC conjugate.

(a) shows immunofluorescent specific to the islet cells obtained with BB rat serum (c) such immunofluorescence was not observed in the islet incubated with control serum (islets are shown by arrows).

(b) and (d) are the phase contrast photographs of the islet shown in (a) and (c) respectively.

DEMONSTRATION OF ICA

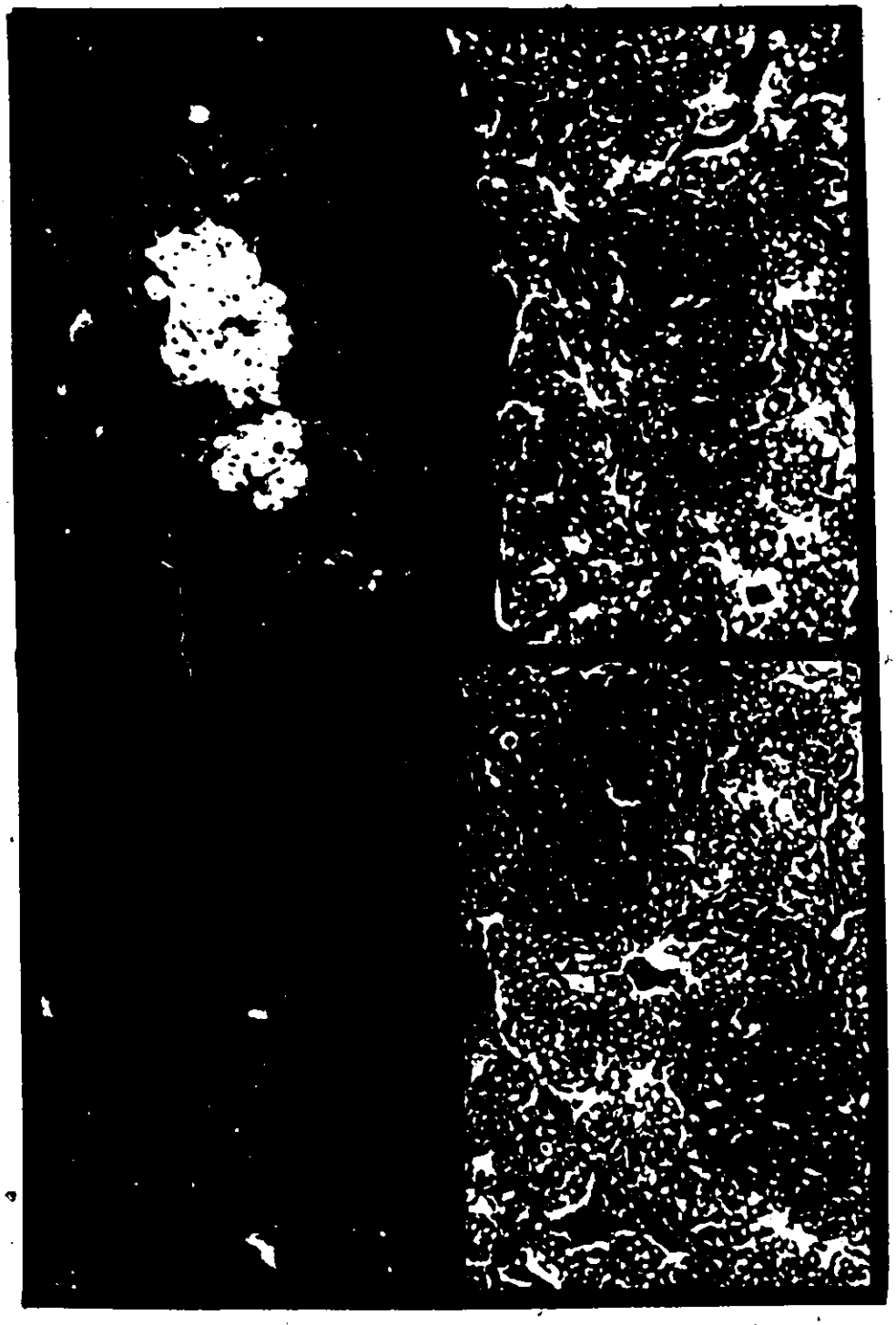


TABLE 2

BLOCKING OF CYTOPLASMIC IMMUNOFLUORESCENCE STAINING OF ISLETS WITH UNLABELLED ANTI RAT IgG

SERUM SOURCE	CONCENTRATIONS OF UNLABELLED ANTI RAT IgG				
	0 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml
41 BB	++	+	+	-	-
45 BB	+	+	+	-	-
CONTROL RAT	-	-	-	-	-
BUFFER	-	-	-	-	-

41 BB was non-diabetic rat; 45 BB was diabetic rat

After incubating the pancreatic sections with the diabetic or control rat serum they were incubated with different concentrations of unlabelled anti-rat IgG prior to incubating with FITC labelled anti-rat IgG.

antibodies followed by incubation with anti-rat IgG FITC conjugate and anti-guinea-pig IgG RITC conjugate. The slides were scored under both fluorescein filter for insulin antibodies and rhodamine filter for ICA. The immunofluorescence pattern of islets showed that the BB rat serum was binding to an identical or at least similar area of the islet as that stained by anti-insulin antibodies (figs. 3a and 3b.).

When the rat serum was applied to the sections presaturated with anti-insulin antibodies or when anti-insulin antibodies were applied to the sections preincubated with the diabetic rat serum, a subsequent simultaneous incubation with RITC labelled anti-rat IgG and FITC labelled anti-insulin antibodies, yielded an immunofluorescence pattern similar to that shown in fig.3. These observations suggest that BB rat immunoglobulins or ICA that bound specifically to B-cell cytoplasmic antigen were not anti-insulin antibodies.

The BB rat sera were titrated at higher dilutions. Since both 1:2 and 1:4 serum dilutions showed strong positive reactions with sections, all the subsequent tests were done using 1:4 serum dilutions. Two control slides were included in each set of tests, one was incubated with negative serum from control rat and the other with PBS buffer.

D. Measurment of ICA Levels using Bouin's fixed pancreatic sections.

Islet cell cytoplasmic antibodies were studied in three groups of rats: newly diagnosed diabetic rats, potentially diabetic young BB rats-(both from the high prevalence BB rat

FIGURE 3

IMMUNOFLUORESCENCE OF ISLET WITH
ICA AND INSULIN ANTIBODIES

A pancreatic section was incubated with an excess of guinea-pig anti-insulin antibodies and BB rat serum (1:4 dil.) subsequently incubated with rabbit anti-rat IgG RITC and rabbit anti-guinea-pig FITC conjugates simultaneously.

a) Cytoplasmic immunofluorescence specific for islet cells obtained with the BB rat serum (photograph taken under RITC filter)

b) Immunofluorescence specific for islet cells obtained with anti-insulin antibodies (photographed under FITC filter). Immunofluorescent patterns obtained with ICA and anti-insulin antibodies are similar.

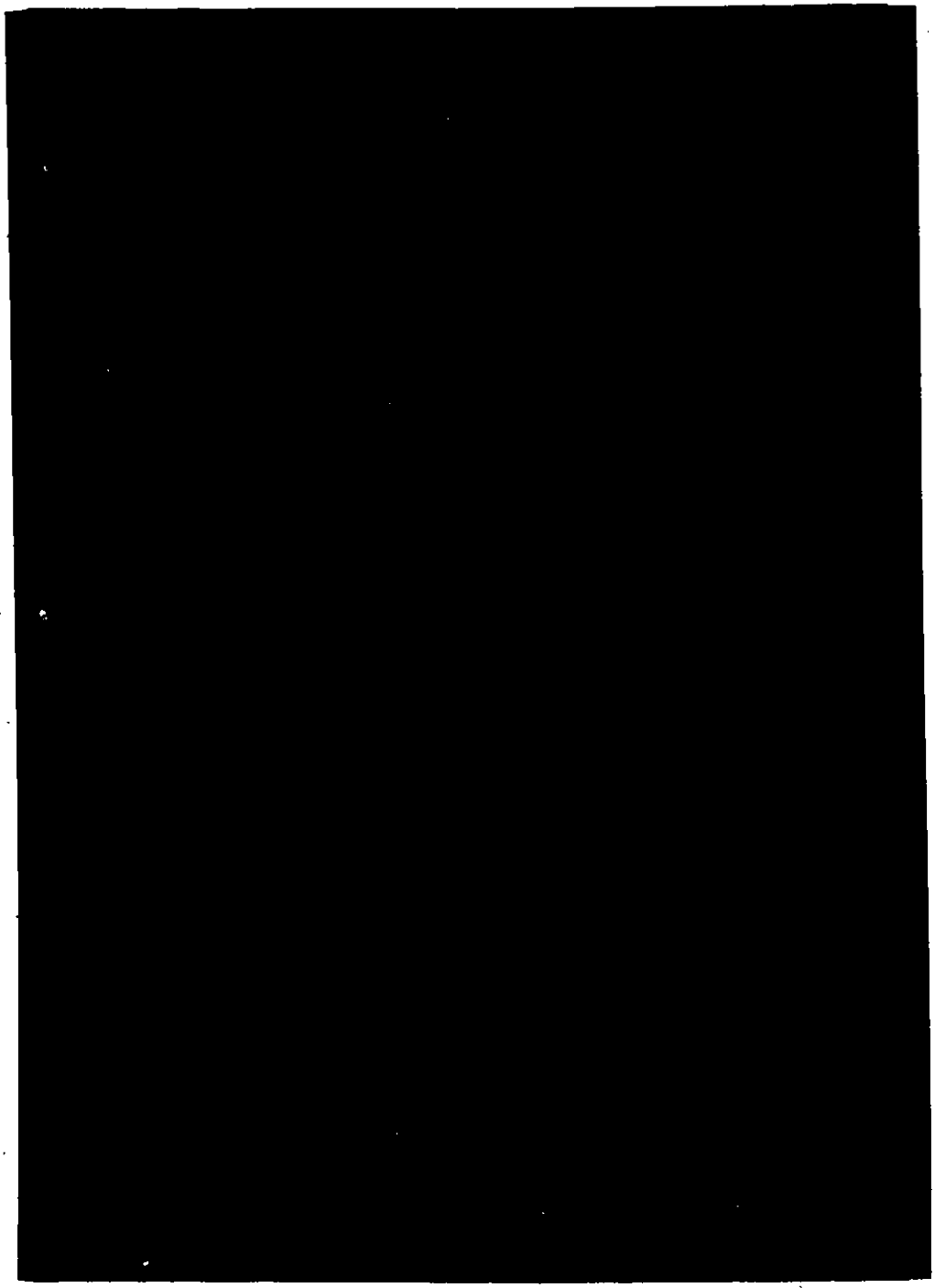
FIGURE 3

IMMUNOFLUORESCENCE OF ISLET WITH

ICA AND INSULIN ANTIBODIES

a

b



colony), and normal healthy rats from the control colony. The group of 22 newly diagnosed diabetics consisted of 5 males and 17 females, twenty one of these developed the overt symptoms between the age of 87-104 days and one female rat developed the symptoms at the age of 137 days. Blood specimen from these animals were obtained between 24 and 72 hr after the detection of glucosuria. The group of 20 potential diabetics consisted of 10 male and 10 female BB rats ranging in age from 44-54 days. The ages of 13 control rats ranged from 85-100 days.

All the slides were scored with a photodetector using the Bioscan software package. The instrument was calibrated against the reference standard as 100% intensity. The intensities of islets and that of exocrine cells (henceforth referred to as background) were read by the photodetector as percentages of the reference standard's intensity. The measurements were averaged over ten observations for every slide. Islet cell fluorescent intensity was calculated as a difference between the total intensity of the islet cells and that of the background. The results are summarized in fig.4. BB rat sera yielded islets of varying immunoflurescent intensity. The values of immunoflurescent intensities of islet cells obtained with diabetic rat sera and potential diabetic rat sera were significantly higher than those for control sera, porabability of error being at 0.1% level ($P<0.001$) for diabetics and at 0.2% ($P<.002$) level for Potential diabetic young rats. Since the values of immunofluorescent intensities are skewed to the left, a non

FIGURE 4

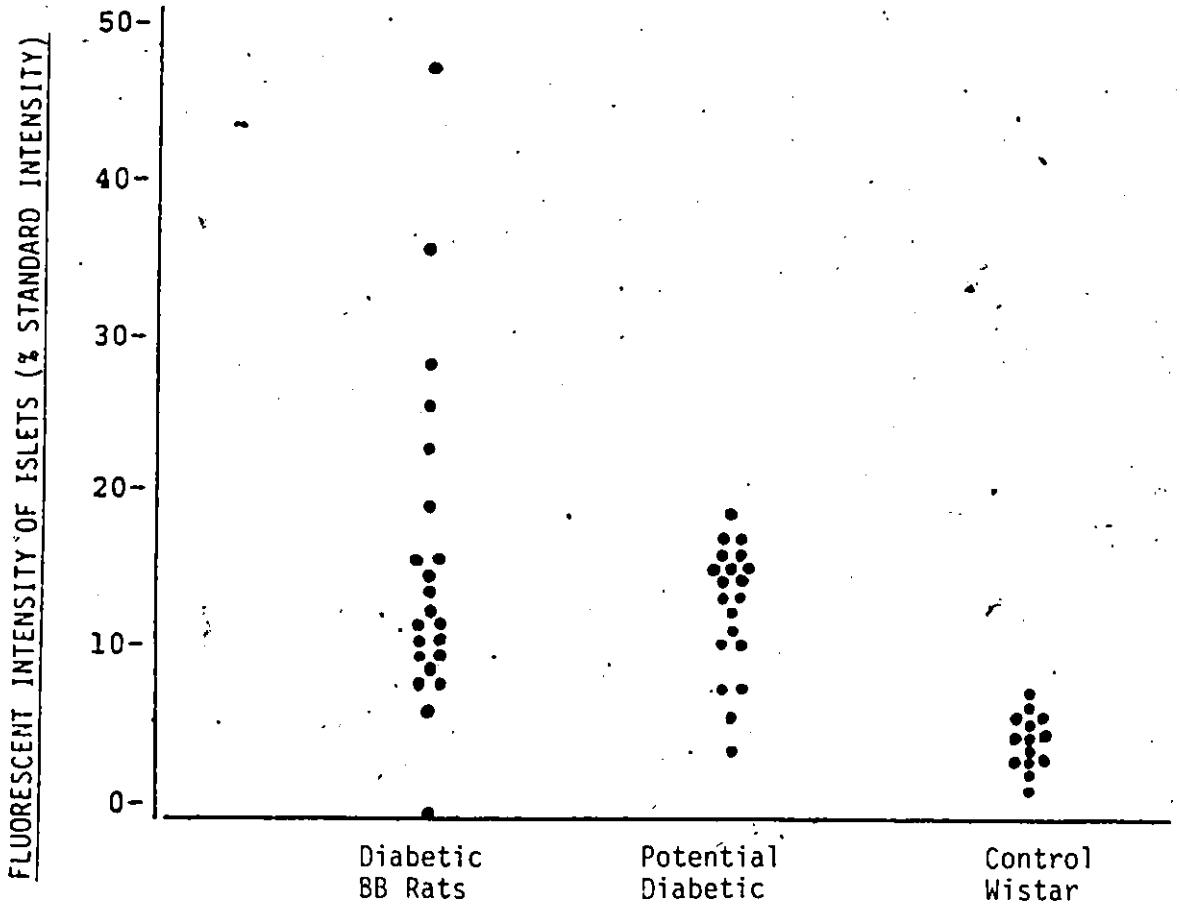
LEVELS OF ICA IN BB RATS

Fluorescent intensity of the islets represents the level of ICA in a serum sample. Fluorescent intensity of the islets is the difference between the total immunofluorescent intensity of the islet and that of the exocrine background, both measured as percentage of the reference standard.

P_1 and P_2 are the respective probabilities that ICA levels in the diabetic and potential diabetic BB rats are not higher than those in control Wistar rats. P values have been calculated using "Wilcoxon rank sum test".

Figure 4

LEVELS OF ICA IN BB RATS



	Diabetic BB Rats	Potential Diabetic BB rats	Control Wistar Rats
Age Range Days	90 - 140	(44 - 54)	(80 - 102)
ICA Levels Range	(-1.11 - 47.4)	(3.6 - 19.1)	(1.7 - 8.1)
Mean	15.63	12.7	4.5
Median	12.2	14.0	4.4

$P_1<0.001$
$P_2<0.002$

parametric test 'Wilcoxon rank sum test' has been used to calculate the probability of error for this data. The results indicate that ICA are present in both diabetic and potential diabetic BB rats. Sera from control rats were negative.

Determination of ICA on Ethanol Fixed Pancreatic Sections

In order to determine if ethanol fixed pancreas can be used to demonstrate ICA in BB rat sera, 12 BB rat serum samples, which were found to be positive on Bouin's fixed pancreatic sections, were tested on ethanol fixed pancreas. Three of the 12 sera were from diabetic rats and the remainig 9 were from potential diabetics. Eight control sera were also included in the experiment. All slides were scored as above. Only two out of the twelve sera showed positive immunofluorescence on ethanol fixed pancreatic sections (fig. 5).

Discussion

Circulating antibodies to gastric parietal cells, smooth muscles and thyroid colloidal antigens have been demonstrated in the BB rat by two other groups (Elder et al.,1982; Like et al.,1982). However these authors reported that they were unable to demonstrate the presence of ICA in BB rat sera. They were using fresh frozen pancreatic sections as substrate, which were either air dried or fixed in acetone at at -20^{o}C.

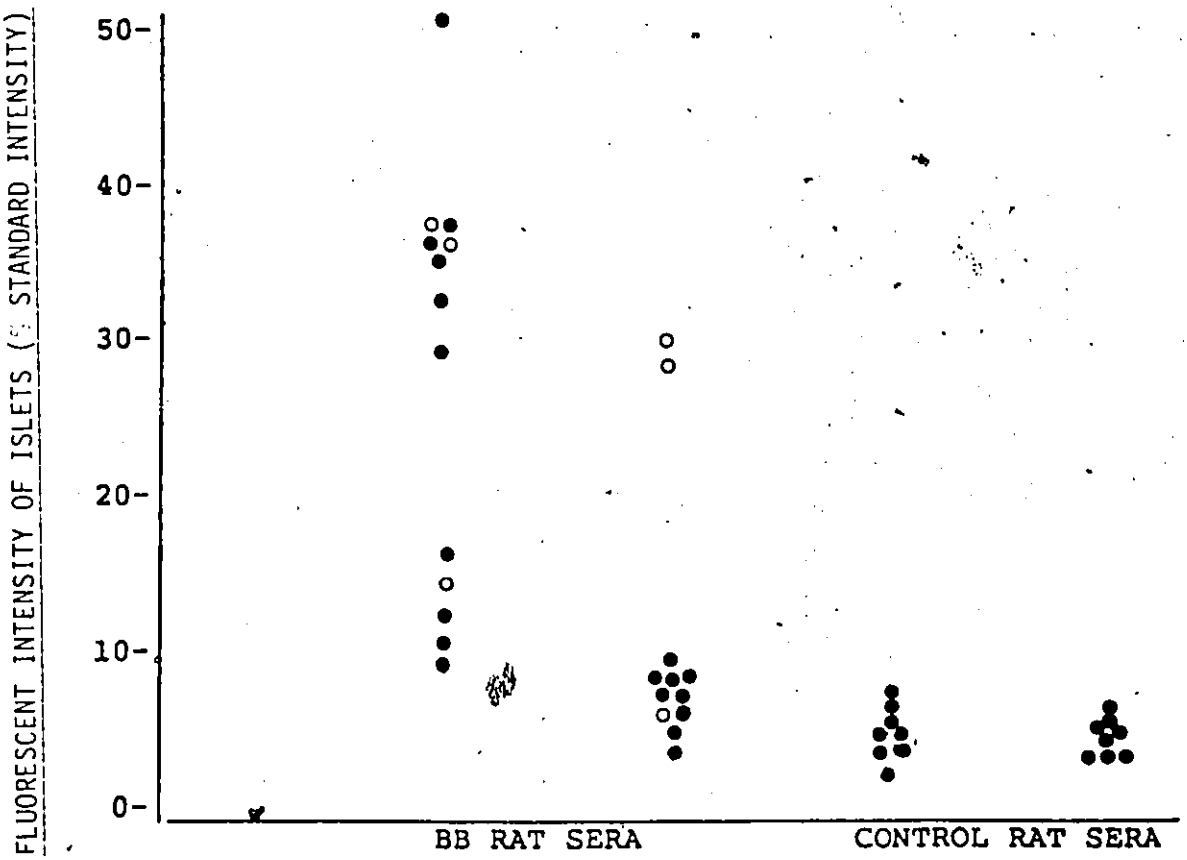
The present study indicates that ICA are indeed present in the BB rat, though, in order to demonstrate the presence of these antibodies Bouin's fixed pancreas had to be used as substrate.In this respect also diabetes in the BB rat resembles the human IDDM. Probably when frozen pancreatic sections were

Figure 5

ICA ON BOUIN'S FIXED PANCREAS

VERSUS

ETHANOL FIXED PANCREAS



o - Samples from diabetic rats.

used the cytoplasmic antigen was washed out of the islet cells during sequential incubations and washings. The unsatisfactory results obtained by us with alcohol fixation are well in line with the negative results observed on acetone fixed sections by the above authors:

The BB rat ICA seem to be speoific for B cells as the distribution of islet cell immunofluorescence obtained with the rat serum closely resembled that observed for intracellular insulin when incubated with anti-insulin antibodies. The histological pattern also showed that these antibodies, unlike ICSA, react with some cytoplasmic determinants of islet cells. The nature of the antigen is not known but it is not insulin because the positive reaction could not be blocked by presaturation with anti-insulin antibodies. However, the reaction was blocked by presaturation with anti-rat IgG; the latter effect was concentration dependent, indicating that rat ICA belong to class G immunoglobulin.

Because ICA could not be detected in the sera from control Wistar rats, we suggest that these are abnormal immunoglobulines. ICA were demonstrated not only in diabetic rats, but also in very young potential diabetic rats (44-54 days old) from the same colony. About 50% of the potential diabetics are expected to develop the disease eventually, but at the time of specimen collection, none of them had clinical symptoms. These experiments suggest that; (i) the presence of circulating ICA is a characteristic feature of all the rats of the BB rat colony studied here whether they develop diabetes

or not and (ii) in all these rats ICA appear at a young age, well before the onset of the clinical symptoms of the disease.

II ISLET CELL SURFACE ANTIBODIES

A. Islet Cell Suspensions

Islet cell suspensions were prepared from islets maintained in cultures for 4-8 days. Integrity of the cultured islets was verified by monitoring the insulin released in the media. Three different cultures of islets were monitored for two weeks and were judged healthy and functional by measuring the insulin release during this period in response to glucose load.

Islets were dispersed using Swim'sM supplemented with 0.2% trypsin. The viability, the total yield of the dispersed cells and the percentage of the single cells present in the suspension were checked routinely. Table 3 shows the sample data from eight cell suspensions chosen randomly. Each suspension yielded 3-4 million cells depending on the number of culture plates used. Viability of the dispersed cells, as determined by Trypan blue exclusion test, was always above 95%. The single cells obtained in most of the suspensions were above 70% of the total cells recovered with some instances of as low as 60-70% of the total yield. Most of the time the olumps observed consisted of two or three cells.

B. Testing Of Islet-cell Suspension for A and B Cells

Islet cell suspensions were tested twice for the presence of insulin producing B cells and glucagon producing A cells by the ^{125}I-protein A binding method. Bouin's fixed cells in

TABLE 3

REPRODUCIBILITY OF ISLET CELL SUSPENSIONS

TEST NO.	NO. OF CULTURE PLATES USED	TOTAL CELL YIELD ($\times 10^6$)	PERCENT SINGLE CELLS	PERCENT VIABILITY
1	7	2.9	71.4	95
2	9	3.6	86.6	96
3	8	2.9	63.0	96.5
4	10	3.4	67.7	97
5	10	2.6	92.9	96.5
6	10	3.7	94.6	97
7	12	4.1	93.2	98
8	13	4.0	79.4	96

Each culture plate contained islets isolated from the pancreata of 10-13 rats. Total cell yield is the total number of cells recovered in a suspension.

microtitre plates were incubated with rabbit-anti glucagon or guinea pig anti-insulin antibodies followed by the incubation with ^{125}I-protein A. The binding of the radio-ligand to the cells incubated with anti-glucagon antibodies and anti-insulin antibodies indicated the presence of both A and B cells in the suspensions (table 4).

C. Comparison of Five Radio-Ligand Methods to Measure ICSA Levels

Five variations of a radio-ligand assay were compared for measuring the levels of ICSA in rat sera. In all five methods either anti-rat IgG or protein A, each labelled with ^{125}I, was used as a radio-ligand. Two samples from diabetic rats and two from control rats at final dilutions of 1:4 were tested by each method. A summary of results is presented in table 5.

In methods I and II sera were tested by measuring the respective percentages of the total ^{125}I-anti-rat IgG and ^{125}I-protein A bound to the islet cells preincubated with sera using one step radio-ligand assay in tubes.

In method III affinity purified ^{125}I labelled anti rat IgG $F(ab')_2$ fraction was used as the radio-ligand. Also, Hanks buffer was replaced by Dulbecco's BSA II (supplemented with 0.09% sodium azide) and all the incubations were done at 8°C.

In method IV the samples were tested in microtitre plates at 37°C, using two step radio-ligand assay. Since protein A has a low binding affinity for rat IgG but a high binding affinity for rabbit IgG (Langone 1978), an additional step was introduced. After incubating with sera the cells were

TABLE 4

TESTING OF ISLET CELL SUSPENSIONS FOR THE PRESENCE OF A AND B CELLS

PERCENTAGE OF ^{125}I-PROTEIN A BOUND TO THE CELLS INCUBATED WITH ANTI-GLUCAGON OR ANTI-INSULIN ANTIBODIES

TEST NO.	RABBIT ANTI-GLUCAGON ANTIBODIES (IgG)	GUINEA-PIG ANTI-INSULIN ANTIBODIES	CONTROL (BUFFER)
1	4.5	30.4	0.55
2	5.2	30.6	0.60

TABLE 5

COMPARISON OF FIVE METHODS FOR ICSA DETERMINATION

SERUM SOURCE	METHODS OF ICSA DETERMINATION I	II	III	IV	V
	PERCENTAGE OF RADIO-LIGAND BOUND TO CELLS				
250 BB	0.57	1.4	1.7	4.7	34.5
251 BB	0.63	1.2	1.9	7.0	35.5
CONT.1	0.36	0.6	0.8	2.9	13.1
CONT.2	0.40	0.8	NA*	1.9	8.5
BUFFER	0.17	0.5	0.9	1.1	5.5

* Not Done

In all methods, dispersed islet cells were used as substrates. Sera from two diabetic rats and two control rats were compared with buffer alone.

The radio-ligand was ^{125}I-rabbit anti-rat IgG in method I, affinity purified F(ab')$_2$ fraction of the same in method III, and ^{125}I-Protein A in methods II, IV, and V.

Methods I, II, and III were one step radio-ligand assays in culture tubes. Method IV is a two-step assay on microtitre plates and method V is a two-step assay in culture tubes. The second antibody in method IV and V was rabbit anti-rat IgG.

Incubating temperature was 37^{o}C for methods I, II, and IV, and 8^{o}C for method III and V.

incubated with rabbit anti-rat IgG followed by the incubation with ^{125}I-protein A.

Method V was the two step radio-ligand assay as above but the test was carried out in tubes. All the incubations and washings were done at 8°C using Dulbecco's BSA-II (supplemented with 0.09% sodium azide) and cells were incubated with rabbit anti-rat IgG before the final incubation with ^{125}I-protein A.

Each of the five methods showed that the percent binding of the respective radio-ligand was higher for BB rat sera than that for control sera or the buffer, indicating that the presence of ICSA could be demonstrated by all five methods. However, method V was found to be the most sensitive for two reasons (i) the percent binding of the radio-ligand obtained for the BB rat sera was much higher than with other methods, and (ii) the difference was largest between the radio-ligand binding for test sera and that for buffer (table 5).

All subsequent determinations of ICSA were done using method (V) and all sera were tested at a final dilution of 1:4. The dilutions were done in Dulbecco's BSA II. Aliquots of sera pooled from two control rats were used as negative control and the aliquots from a diabetic rat serum showing high binding to the cells were used as a positive control in each set of tests. The same controls were used throughout the study.

Since no significant difference was observed between the binding of commercially labelled protein A and the protein A

labelled in this laboratory, commercially iodinated ^{128}I-protein A was used for all subsequent tests.

D. Measurment of Levels of ICSA Binding

Islet cell surface antibodies, like ICA, were studied in three groups of rats; (i) newly diagnosed diabetic BB rats, (ii) non diabetic young BB rats referred to as potential diabetics and (iii) normal healthy rats from our control colony. The group of 26 newly diagnosed diabetic rats consisted of 13 females and 13 males with ages ranging from 87 to 137 days. Blood from these was sampled between 24 and 72 hours after the detection of glycosuria. The group of 26 potential diabetic rats also consisted of equal number from both sexes with ages ranging from 44-80 days. The ages of 16 control animals ranged from 85-100 days.

ICSA levels in sera were determined by method V. The percentage of the total radioligand bound for the sample (after subtracting the non specific binding for buffer control) was the measure of ICSA levels. The results summarized in fig.6 are the mean of duplicate determinations. The observation indicate that ICSA, like ICA, are present in both diabetic and potential diabetic BB rat sera.

^{128}I-protein A binding levels for sera from diabetic as well as potential diabetic BB rats were significantly higher than those obtained for control sera. The probability that ICSA levels in the BB rats (each group diabetic and potential diabtic) are not higher than those in the control is at 0.1% level ($P < 0.001$, as calculated by 'Wilcoxon rank sum test').

FIGURE 6

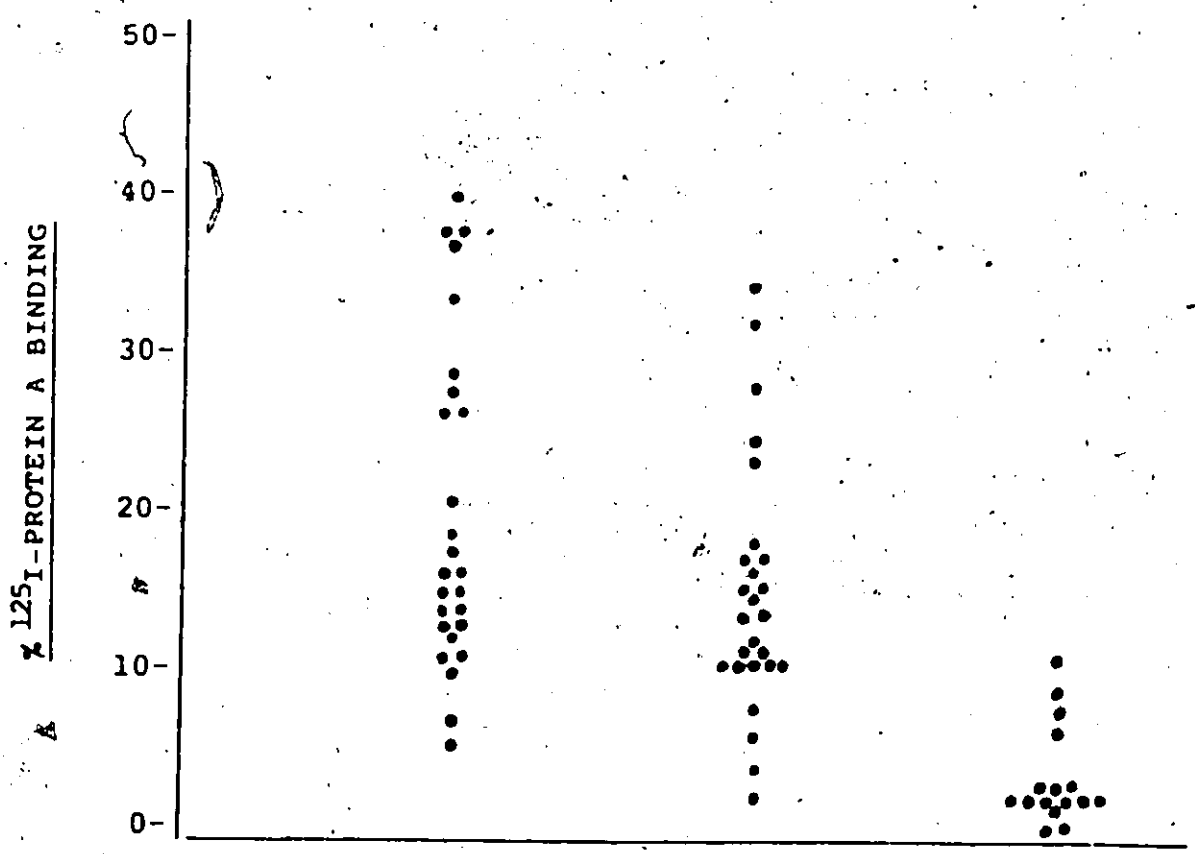
LEVELS OF ICSA IN BB RATS

Percentage of the total radioligand bound to the islet cells for a serum represents the ICSA levels in the serum. Radioligand binding for serum has been calculated after subtracting the nonspecific binding for the buffer.

P_1 and P_2 are the respective probabilities that ICSA levels in the diabetic and potential diabetic BB rats are not higher than those in control Wistar rats. P values have been calculated using "Wilcoxon rank sum test".

Figure 6

LEVELS OF ICSA IN BB RATS



	Diabetic BB Rats	Potential Diabetic BR Rats	Control Wistar Rats
Age Range Days	(90 - 140)	(44 - 80)	(85 - 102)
ICSA Levels Range	(5.9 - 40.2)	(23 - 34.0)	(-0.1 - 11.0)
Mean	20.3	14.6	3.5
Median	16.1	13.1	2.3

$P_1 < 0.001$
$P_2 < 0.001$

E. Affinity of ICSA for Spleen Cells

Since Dyrberg et al (1984) reported that BB rat ICSA can be absorbed with spleen cells, we studied the affinity of ICSA for spleen cells by comparing ^{125}I-protein A binding for the samples absorbed with spleen cells versus unabsorbed samples. Both the BB rat sera and control sera were tested. The results are summarized in table 6. No significant difference was observed between the ICSA levels for unabsorbed sera and the sera absorbed with an equal volume of packed spleen cells. Contrary to Dyrberg's findings, in these experiments ICSA could not be absorbed out of sera with spleen cells.

F. Complement Dependent Cytotoxicity Of Antibodies

Cytotoxicity of antibodies to islet cells in-vitro was studied by measuring the amount of ^{51}Chromium released from the cells by the serum in the presence of complement. Sera from five diabetic BB rats aged 102 days and from five control rats aged 85 days were used for the experiment. The sera from diabetic rats were sampled 24 hours after the onset of overt symptoms. As shown in table 7, considerable release of ^{51}Cr was observed in both groups and no significant difference was found between the release of ^{51}Chromium by diabetic sera or control sera in the samples studied.

Discussion

The presence of ICSA has been demonstrated earlier in this laboratory (Pollard et al. 1983) by an indirect immunofluorescence method and by Dyrberg et al.(1984) using a semi-quantitative technique, based on ^{125}I-protein A binding.

TABLE 6

COMPARISON OF ISLET CELL SURFACE ANTIBODIES IN UNABSORBED SERA AND SERA ABSORBED WITH RAT SPLEEN CELLS

TEST NO.	SOURCE OF SERUM	PERCENT RADIOLIGAND BOUND TO THE CELLS	
		INCUBATED WITH UNABSORBED SERUM	INCUBATED WITH ABSORBED SERUM
1	179 BB	10.4	12.8
2	180 BB	17.7	13.8
3	181 BB	10.7	11.5
4	182 BB	9.6	9.4
5	33 BB	15.9	25.0
6	43 BB	22.2	24.9
7	211 BB	11.5	9.5
8	212 BB	13.0	9.4
9	227 BB	30.8	26.3
10	228 BB	11.2	10.3
11	234 BB	15.7	12.0
12	236 BB	15.1	13.7
13	185CONT	4.6	3.6
14	186CONT	7.7	3.4
15	187CONT	3.6	2.8
16	188CONT	7.8	3.2
17	234CONT	8.5	8.7

Absorbed sera were prepared by incubation with equal volumes of rat spleen cells for 45 min. at 37°C.

TABLE 7

COMPLEMENT DEPENDENT CYTOTOXIC ANTIBODIES MEASURED BY THE CHROMIUM RELEASE METHOD

SERUM SOURCE	PERCENT ^{51}CHROMIUM RELEASED		AVERAGE CHROMIUM RELEASED X±S.D.
	SERUM CONTROL	SERUM + COMPLEMENT	
179 BB	7.8	38.0	
180 BB	10.1	40.5	
181 BB	8.8	48.1	41.0 ± 5.5
182 BB	9.9	44.4	
183 BB	6.2	34.0	
184 CONT	9.2	40.9	
185 CONT	8.7	50.6	42.8 ± 4.4
186 CONT	11.7	39.8	
187 CONT	7.1	40.7	
188 CONT	10.9	42.4	
COMPLEMENT CONTROL Buffer + complement		15.2	

All the BB rats were newly diagnosed diabetics. Each individual result in the Table represents an average of 3 duplicate assays.

Dyrberg et al used extremly high quantities of radioactivity (Approx. $1x10^6$ cpm per tube.) because only a minute fraction of the total ligand added (less than 1%) was bound to the cells. Most likely, the low binding of the ligand in their experiment was due to the low binding affinity of protein A for rat IgG (Langone, 1978). We tried to circumvent the problem in two different ways: by using ^{125}I labelled anti-rat IgG instead of protein A, and by developing a two step radioligand assay, i.e., method V. In this method, after rat IgG was bound to the islet cell surface (after incubating the cells with rat sera), the cells were first incubated with unlabelled rabbit anti-rat IgG and then with ^{125}I-Protein A. The two step method carried out at 8^oC proved to be a better method as the binding of the radio-ligand for diabetic rat sera was increased from 1.2% and 1.4% obtained by method II to 34.5% and 35.5% (method V). The attempt to adapt this method for microtitre plate was not very successful. Radio-ligand binding in microtitre plates was probably low because dispersed islet cells maintained in microtitre plates tend to clump together forming pseudo-islets, thus reducing the cell surface area avilable for reaction with serum.

In method III we tried an affinity purified preparation of rabbit anti-rat IgG, believing that the unpurified anti-rat IgG used in method I most likely contained antibodies to various other antigens, which would result in low binding of the tracer. Although the use of affinity purified IgG inoreased the percentage of the ligand bound to the cells, the

sensitivity of this method was still much lower than that of method V. Therefore method V was chosen for subsequent tests.

The results of this study have confirmed our earlier observations obtained by immunofluorescence method (Pollard et al.1983) that ICSA could be detected in the sera of all the animals from the BB rat colony whether they develop diabetes or not. Furthermore these antibodies could be detected in circulation long before the onset of the disease.

Our failure to show the binding affinity of ICSA to spleen cells can not be explained. The discrepancy between the results of this study and that reported by Dyrberg (1984) could be due to the different methods used for preparing spleen cells. Also, the fact that the original BB rat colony was not inbred and allowed for considerable genetic heterogeneity should be considered. Various BB rat subcolonies now maintained at different locations, inbred or partially outbred, have been derived from only a few animals from the original colony. This could introduce some genetic difference among these subcolonies.

The experiments with complement dependent cytotoxicity were rather frustrating. The results for the same set of sera obtained with different batches of complment were not reproducible. The results shown in table 7 are only an example from one set of experiments. No reliable conclusion could be formulated.

III. Longitudinal Study of ICA and ICSA

The levels of ICA and ICSA were followed in weekly samples drawn from 20 BB rats (an equal number from both sexes), starting at the age 6-7 weeks, i.e. well before the earliest onset of the overt symptoms. In sixteen of these animals islet cell antibodies were followed until their death and in the remaining four the longitudinal study was terminated after 30 weeks. Nine of the twenty animals, 7 females and 2 males, became overtly diabetic during the study. Six of the seven diabetic females came from the same litter.

ICA levels in the samples drawn on the same day from all the animals were tested in one assay. As far as possible ICSA determinations in all the serial samples from one rat were done in one assay to eliminate inter-assay variation. However, in case of three animals the number of samples was too large to be analysed in one assay. Therefore, the samples from each of these three animals were analysed on two different days, repeating some of the samples from previous day. All determinations were done in duplicate. Twenty one samples and three controls could be easily handled in one assay. Reproducibility of ICSA assay was checked by the ratio of positive and negative control. The mean and standard deviation for 28 tests was 3.4+ 0.4 with a coefficient of variation 11%. Intra-assay variation was checked by taking eight measurement for one sample. The mean and standard deviation was 84.2±2.5.

Both ICA and ICSA were found in all the animals whether they developed clinical symptoms or not (fig. 7 & 8).

Considerable fluctuations were observed in the binding levels of both ICA and ICSA during the course of the study. The changes in the levels of the two antibodies were mostly in the same direction, however, in some instances it was observed that the level of one type of antibodies was increasing, while that of the other type was decreasing.

Fig.7 and Fig.8 show representative examples from four animals that became diabetic and four that did not become diabetic during the study. The observations suggest that neither the time of onset nor the clinical symptoms of diabetes influence the levels of either type of antibodies. However, it appears that the mean level of ICSA gradually increases with age in the BB rat studied here (fig.9).

In a few instances the antibodies, more often ICA than ICSA, were undetectable in one specimen but reappeared in samples from subsequent weeks.

DISCUSSION

The longitudinal study confirmed, as well as complemented, the results reported in the first two parts of this study. All the animals from the diabetes prone BB rat colony were found to develop both ICA and ICSA early in life. About one half of these animals became diabetic, but neither the sudden drop of insulin levels nor the profound metabolic changes accompanying the acute ketoacidosis have a major effect on the levels of circulating antibodies. Yet neither ICA nor ICSA could be detected in sera from control Wistar rat colony derived from the same original Wistar colony at BioBreeding

FIGURE 7 and FIGURE 8

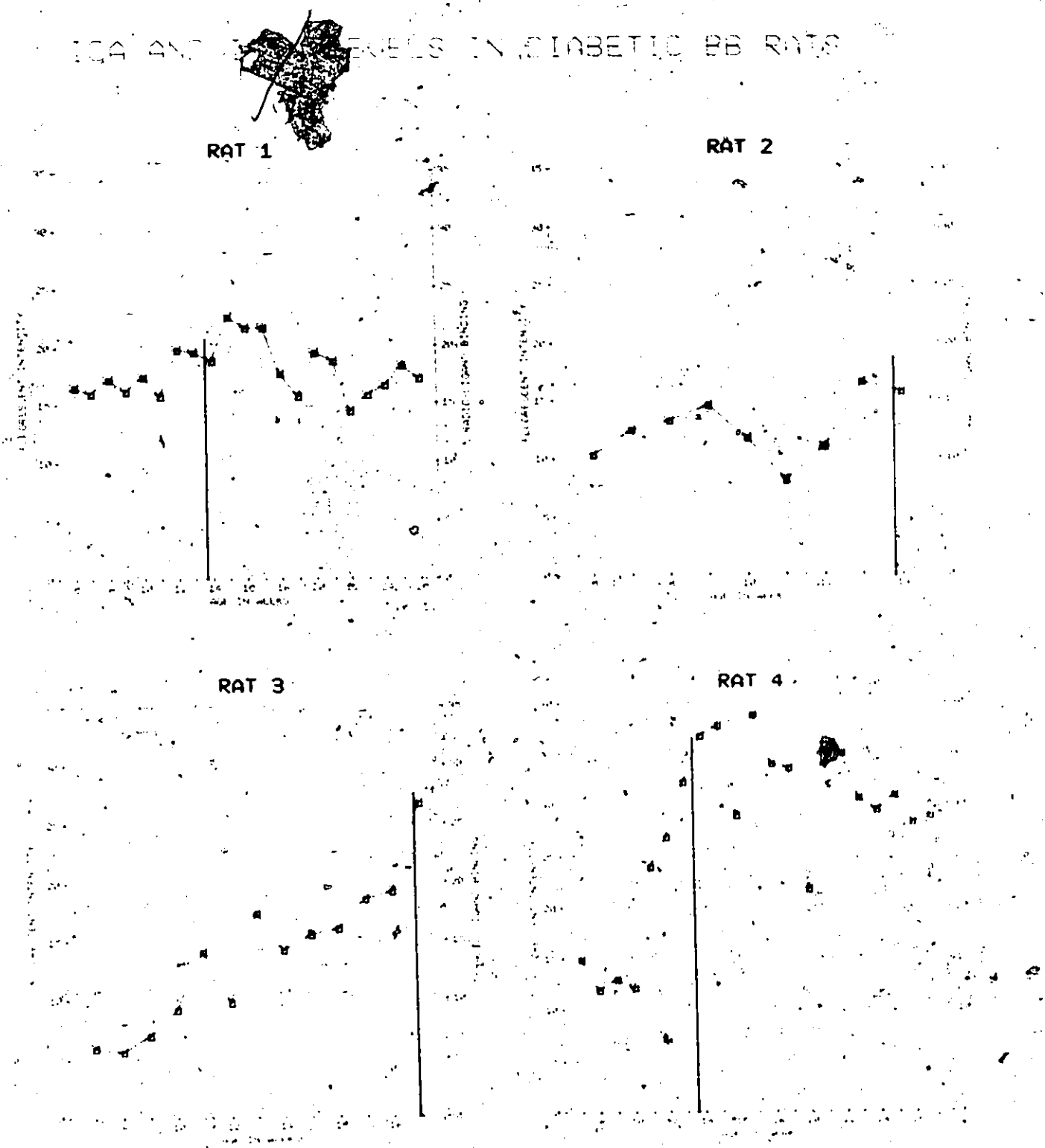
ICA AND ICSA LEVELS IN DIABETIC
AND NON-DIABETIC BB RATS

Fluorescent intensity of the islet represents the difference between the total intensity of the islet and that of the exocrine cells, both read as % of reference standard.

% binding of radio-ligand represents the ^{125}I-Protein A binding for sera after subtracting the non-specific binding.

FIGURE 7

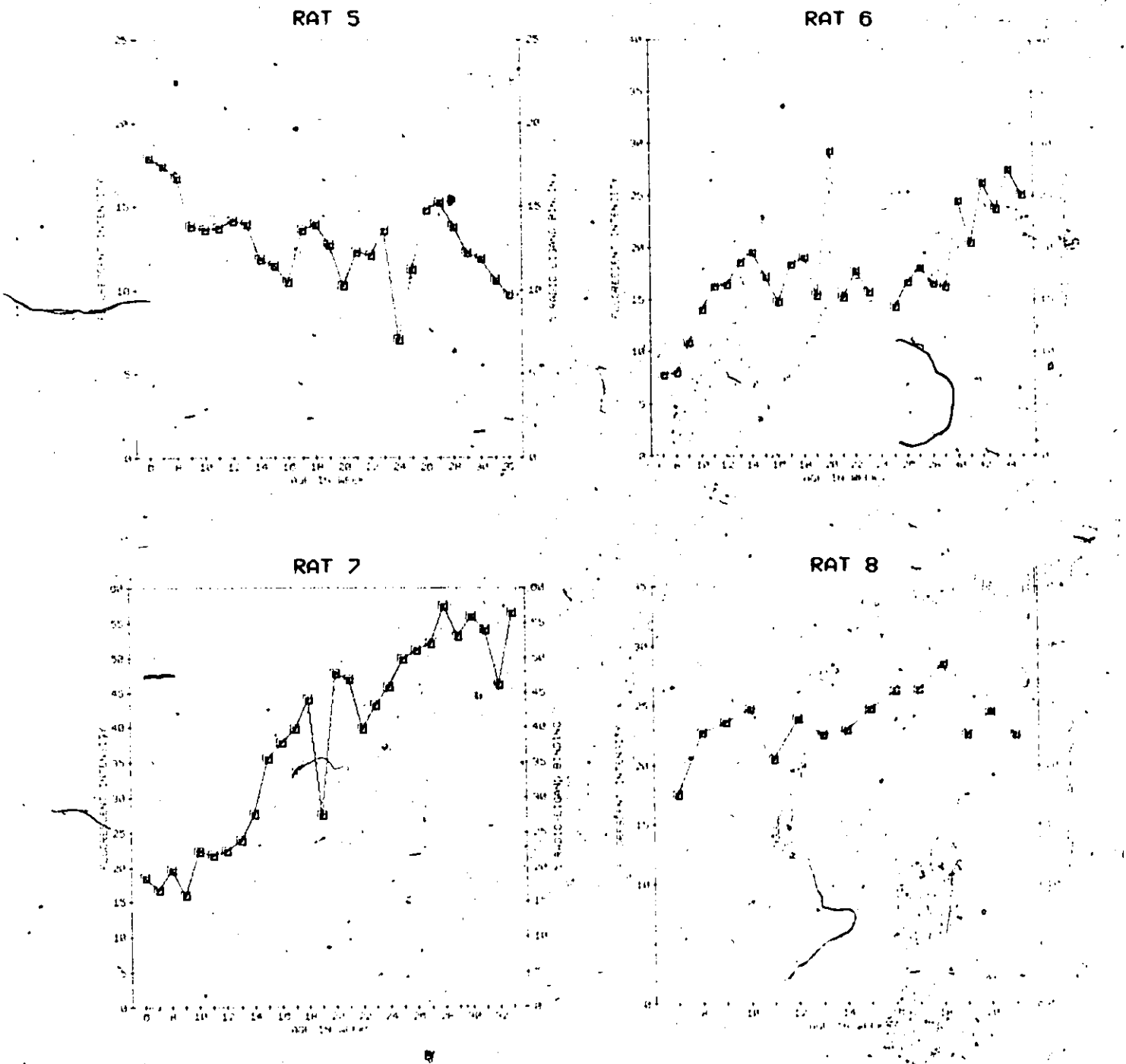
ICA AND ICSA LEVELS IN DIABETIC BB RATS



ICSA ⊟ ICA ▽

- Vertical line represents the age of onset of diabetes
- ICSA level for control serum: Mean + 2SD = 9.7
- ICA levels for control serum: Mean + 2SD = 7.3

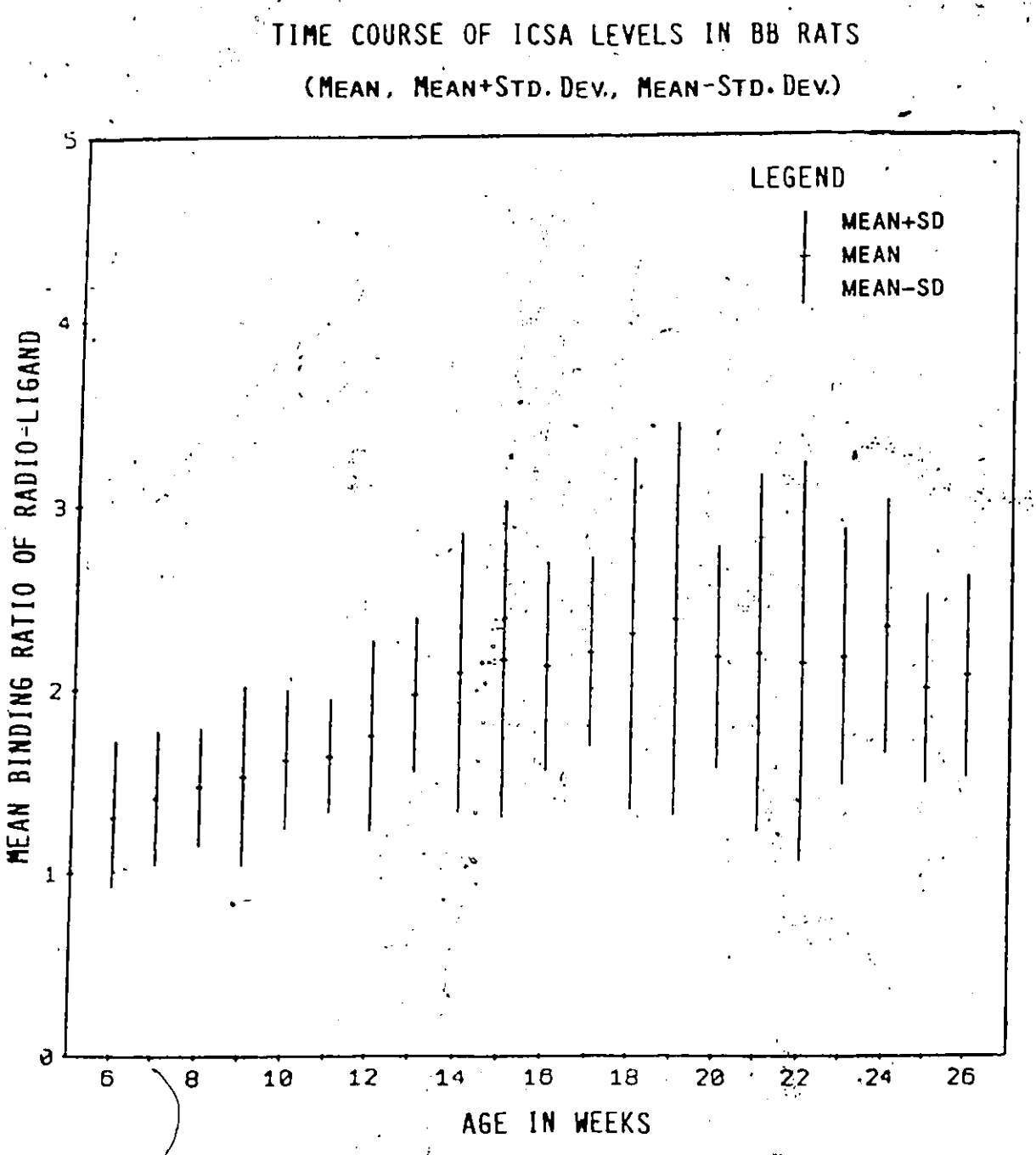
FIGURE 8



ICSA ICA

- ICSA level for control serum: Mean + 2SD = 9.7
- ICA levels for control serum: Mean + 2SD = 7.3

FIGURE 9



Mean levels of ICSA in BB rats are represented by the mean binding ratio of the radio-lagand, ^{125}I-Protein A. The binding ratio of the radio-ligand is the ratio of ^{125}I-Protein A bound to the cells for test serum and that for the negative control. From weeks 6-10 the specimens are from 16 BB rates (N = 16), but the number of specimens gradually decreases to 10 by week 26, as some of the animals died during the period of study.

Laboratory. Whether these antibodies play any pathogenic role in the development of the disease is not determined. The antibodies may be simply a byproduct of an underlying genetic disorder.

The potential importance of these observations in my opinion is two-fold: (i) Islet cell antibodies are the true genetic marker of the diabetes prone condition in the BB rat. Inheritance of diabetes in the BB rat does not show a clear mendelian pattern. A two gene theory has been purposed to explain the observed pattern of the disease (Colle et al.,1983). On the otherhand there is a strong indication that the susceptibility to the disease in the BB rat is a result of the single gene mutation in a Wistar rat colony, because:(a) the disease appeared suddenly in only one Wistar colony,(b) it steadly reappeared in the offsprings, (c) and there is a high prevalence of the disease. However, these observations do not rule out the possibility of the requirement of the second gene, presumably quite prevalent in Wistar rat, for the expression of the disease. In either case the immune system of the BB rat seems to be affected by the primary hereditory condition, as ICA and ICSA are present in all the animals of the BB rat colony. In that case detection of islet cell antibodies, instead of clinical symptoms of diabetes, can be used to study the genetics of the BB rat.

(ii) There are reports of higher prevalence of circulating islet cell antibodies in the first degree relatives of diabetic probands than in general population. These

observation suggest that even in man, IDDM may be an expression of a severe autoimmune condition. Whereas, the clinical symptoms of diabetes may remain unexpressed in milder cases of the autoimmune condition.

CONCLUSION

It is shown that not only the antibodies to pancreatic islet cell surface (ICSA) but also the antibodies to islet celll cytoplasmic antigens (ICA) are present in the diabetes prone BB rat. Both ICA and ICSA are present in all the animals of the BB rat colony studied, whether they develop diabetes or not, and the antibodies are detectable in fairly young animals (7 weeks old). However neither of the two antibodies could be detected in any of the control Wistar rats.

There is no correlation between the levels of either of the antibodies and the time of onset of the disease. In fact the levels of both ICA and ICSA fluctuate considerably in an unpredictable manner during the life time of an individual animal whether the animal became diabetic or not.

It was observed that some times one type of antibodies may even disappear from circulation temporarily, while the other was still present at high level, indicating that two different antigens are involved in this autoimmune condition.

It appears that the presence of these antibodies is a true immunological marker of the genetic abnormality in the BB rat. Development of diabetes in only 40-70% of the animals of this colony suggests that overt symptoms of diabetes are expressed only in the severe condition of an underlying autoimmune disorder.

It will be interesting to investigate if the antigens in question can be detected in circulation as the free entities or complexed with the respective antibodies.

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