AUTOAGGRESSIVE T CELLS IN INSULIN-DEPENDENT DIABETES MELLITUS

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ABSTRACT

Considerable evidence suggests that insulin-dependent diabetes mellitus (IDDM) is a T cell-mediated autoimmune process that is directed against antigenic target(s) on pancreatic β cells. To better understand T cell involvement in the pathogenesis of IDDM, a panel of T cell hybridomas were produced from pancreas-derived T cells of spontaneously diabetic NOD mice. A total of 119 hybridomas were constructed from 8 fusions and 94 of these were tested. Twelve hybridomas were found to be islet-reactive since they produced high level of interlukin-2 (IL-2) in the presence of NOD islet cells and NOD antigen-presenting cells (APC's). The responses could also be detected against islet cells of other strains (i.e, C3H/Hej or C57Bl/6), but only in the presence of the NOD APC's. Phenotyping of these islet-reactive hybridomas showed that all of them were $CD3^+CD4^+$. Furthermore, a high frequency (39%) of $CD4^+$ T hybridomas in the panel were found to be islet reactive. In addition, analysis of T-cell receptor (TCR) V_β expression of these islet-reactive T-cell hybridomas revealed that TCR V_β element usage is hecerogeneous unlike findings in some experimentally induced autoimmune diseases.

RESUME

Plusieurs évidences tendent à suggérer que le diabète sucré insulino-dépendant (DSID) est un procedé autoimmun généré par des lymphocytes T dirigés contre une ou des cibles antigeniques des cellules Bêta du pancréas. Pour mieux saisir l'implication des lymphocytes dans la pathogenèse du DSID, un panel d'hybridomes T fut constitué à partir de lymphocytes T provenant du pancréas de souris NOD spontanément nouvellement diabétiques. De 119 hybridomes, provenant de huit fusions, 94 furent testés. De ceux-ci, 12 hybridomes se sont avérés réactifs envers les ilôts pancréatiques de souris NOD et envers les cellules présentatrices d'antigènes (CPA) de même origine. Des taux élevés d'interleukine-2 (IL-2) ont été produits en leur présence alors que les échantillons-contrôles n'induisaient que de faibles taux d'IL-2. Ces mêmes hybridomes réagissent contre les cellules d'ilôts pancréatiques de différentes lignées de souris (e.g., C3H/Hej ou C57BL/6) mais, seulement en présence de CPA de souris NOD. Le phénotype des hybridomes réactifs indique qu ils sont tous $CD3^{+}CD4^{+}$. De plus, un pourcentage important (39%) des hybridomes-T $CD4^{+}$ du panel réagissent contre les cellules d'ilôts. Enplus, l'analyse de l'expression des chaînes-β des récepteurs cellulaires-T (RcT) revèle que leur utilisation par les hybridomes reactifs est hétérogène, contrairement à certaines maladies autoimmunes induites expérimentallement.

Traduit par H. Ste-Croix

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PART 1. INTRODUCTION AND LITERATURE REVIEW

I. INTRODUCTION

Diabetes mellitus represents a heterogeneous group of disorders where an absolute or relative deficiency of insulin results in impaired carbohydrate, fat, and protein metabolism (Volk, 1985). Diabetes mellitus ranks among the top ten causes of death in Western nations, and is one of the most common metabolic diseases in humans. Despite important improvements in its clinical management, it has not yet been possible to prevent several diabetes-related lesions.

Until recently, several classifications have existed based on differing criteria (some using clinical features, others etiology and some the presumed natural history of diabetes). In an attempt to overcome problems in classification, the National Diabetes Data Group of the National Institutes of Health developed a classification; which basically divides spontaneous idiopathic diabetes into two types (Bennett, 1983): Type I (insulin-dependent diabetes mellitus, IDDM) and Type II (non-insulin-dependent diabetes mellitus, NIDDM). Patients with diabetes secondary to surgery, pancreatitis, or other diseases are placed in a separate category.

Type I diabetes (IDDM) is also called juvenile onset or ketotic diabetes. It accounts for approximately 10% cases of diabetes mellitus (the other 90% being predominantly type II diabetics). Type I diabetes usually develops in childhood or puberty and is characterized by severe

hyperglycemia and complications such as ketoacidosis and coma. Type I diabetes results from a severe lack of insulin, caused by a selective and irreversible destruction of insulin-producing pancreatic β cells. Patients depend on insulin therapy for survival. Three interlocking mechanisms are believed to be responsible for the β cell destruction: genetic susceptibility, autoimmunity, and an environmental insult. These three factors influence the nature and course of IDDM and contribute to its complex pathological picture.

Type II diabetes is also called adult-onset or nonketotic diabetes. This type occurs at any age but usually appears in the older obese patient. It is characterized by partial insulin deficiency and in most patients there is insulin resistance at target tissues. This type will not be discussed further in this thesis as my work is on IDDM.

To study the pathogenesis of human type I diabetes, several animal models have been used for several years: the BB rat model (Chappel and Chappel, 1983), the NOD mouse model (Makino et al., 1980), and mice treated with multiple sub-diabetogenic doses of the drug streptozotocin (Like and Rossini, 1976). Additional important data have also been provided by the recent creation of unique transgenic mice (Lipes and Eisenbarth, 1990).

IDDM is now widely believed to be a chronic autoimmune disease in which activated T-lymphocytes invade and destroy the insulin producing β-cells of the pancreatic islet. This view is strongly supported by studies on BB rats and NOD mice models, both of which spontaneously develop IDDM. However, the exact pathogenesis of Type I diabetes is

still not clear. It seems that these activated T-lymphocytes in the islets are pathogenic, like T-cell clones isolated from other autoimmune diseases such as experimental allergic encephalomyelitis (EAE) (Ben-Nun et al., 1981) and autoimmune thyroiditis (Pontes de Carvalho et al., 1981; Rombal and Weigle, 1987). Thus the identification of islet-specific T-cell clones may lead to a better understanding of the pathogenesis of diabetes and novel therapeutic approaches for treatment or prevention of the disease.

II INSULIN-DEPENDENT DIABETES MELLITUS (IDDM)

II.1. IDDM IN HUMAN

A. HISTORY

Diabetes mellitus has been known to man since ancient times. To explain the origin of diabetes mellitus, Von Mering and Minkowski first produced experimental diabetes by removing the dog's pancreas in 1889 (Von Mering and Minkowski, 1889; Minkowski, 1893). It was concluded that diabetes mellitus was associated with a hitherto unknown function of the pancreas. A likely connection to diabetes was evidenced by Dieckhoff's observations in 1894 that the pancreas of diabetic patients had a greatly diminished number of pancreatic islets (Dieckhoff, 1894). Technical advancements, particularly in techniques of microscopy and histology, allowed Lane (1907) and Bensley (1911) to describe the pancreatic A and B cells, and Meyer (1909) proposed insulin as the factor from the pancreatic islets that controlled blood sugar (see: Lernmark and Baekkeskov, 1986). A search for insulin was undertaken by

many workers; it often failed, however, due to the extensive proteolytic activity in pancreatic extracts and the specific requirements to solubilize insulin. It was not until 1921 that Banting and Best successfully prepared pancreatic extracts containing sufficient amounts of biologically active insulin in Toronto (Bliss, 1982). The etiology and pathogenesis of the disease, however, remained unknown.

In 1965, Gepts (1965) performed a careful morphological investigation of the pancreas of a newly diagnosed IDDM patient and found major abnormalities in a number of B cells (i.e, β cells) and the presence of inflammatory cells in the islet of Langerhans (insulitis). The observation of insulitis provided the earliest morphological clue to the pathogenesis of IDDM. Subsequent data have demonstrated that autoantibodies directed against both islet surface and cytoplasmic antigens are present in affected individuals before diabetes occurs (Bottazzo et al., 1974) and a strong association of disease expression with certain class II major histocompatibility complex (MHC) antigens (Todd, 1990. Review). Interestingly, Nerup (1971) and Boitard et al., (1981; 1982) showed that T cells from diabetics could suppress the release of insulin by islet cells *in vitro*, and moreover, islet-specific T-cell lines or clones have recently been isolated from both animal models (Prud'homme et al., 1984; Haskins et al., 1988; Reich et al., 1989a) and humans (Vliet et al., 1989). Furthermore, several recent clinical trials have demonstrated that systemic immunosuppression with cyclosporine (Bougneres et al., 1988) or azathioprine and prednisone (Silverstein et al., 1988) can substantially ameliorate the syndrome, greatly prolonging the "honeymoon" period of remission. It has also been

observed that when a pancreas is transplanted from a non-diabetic to a diabetic monozygotic twin in the absence of immunosuppression, the islets are destroyed (Sutherland et al., 1984). This result indicates that the immune system of type I diabetes can still recognize and destroy normal β cells, even several years after onset of disease.

According to these studies during the past two decades, IDDM has been classified as an autoimmune disease. However, the precise mechanisms that result in immune-mediated β cell destruction in IDDM remain unknown.

B. CLINICAL SIGNS AND COMPLICATION

The chief signs and symptoms of untreated diabetes are polyuria, glycosuria, high blood sugar, excessive thirst and hunger, marked weakness, and loss of weight caused by deranged carbohydrate metabolism. Other symptoms due to the incomplete combustion of fats are manifestations of acidosis. (e.g., air hunger, coma, and ketone bodies in the urine). Before the advent of insulin therapy, the diabetes syndrome was a fatal disease and lead to a rapid death. There was no remedy until 1921 when insulin was discovered by Banting and Best. Although the insulin injections now prevent the deaths from diabetic ketoacidosis that were formerly inevitable, this therapy does not prevent the late complication of IDDM. These include blindness, renal failure, neuropathy, and peripheral and coronary vascular disease (Rossini, 1980).

C. TREATMENT OF IDDM WITH INSULIN AND IMMUNOSUPPRESSIVE AGENTS

Before 1922 the life expectancy of a child or young adult with diabetes was less than 1 year from diagnosis. By 1924 the life expectancy had risen to 7-8 years, and it improved rapidly with increased knowledge of insulin action. Thus, insulin had a marked social impact; it was long thought to be the cure for diabetes. However, more than 60 years of insulin therapy have proved the hormone can only maintain survival in a chronic disorder, associated with a hypogylcemia potentiality and a 200 to 300% overmortality (Lernmark and Baekkeskov, 1986).

Beside insulin injection, several other treatments have been developed based on the knowledge about immunoabnormalities in IDDM (for review see: Castaño and Eisenbarth, 1990). The most promising results have been achieved with the immunosuppressive agent cyclosporin A (cyclosporine), which maintains continued insulin secretion (e.g., C-peptide secretion) and probably prevents further β cell destruction. However, cyclosporine cannot maintain a nondiabetic state over time when only few β cells are present at onset of diabetes and it is also associated with nephrotoxicity and other side effects. Other immunomodulatory and immunosuppressive agents such as levamisole, plasmapheresis, gammaglobulins, nicotinamide and interferon have also been used both in human and in animal models. Unfortunately, they are less effective in human Type I diabetes.

Despite these and other treatments (including diet and islet or pancreas transplantation), there is not yet any accepted safe way to

prevent the disease or to alter its course once it has begun.

II.2. ANIMAL MODELS OF SPONTANEOUS IDDM

Since the genetic predisposition, etiology, physiology, therapy, and prevention of human IDDM are still not well understood, animal models can provide valuable insights to these unanswered questions.

A. BB RAT

The BB rat, which displays insulin-dependent diabetes with insulitis, was discovered in an outbred Wistar colony at the BioBreeding Laboratory of Canada Ltd., hence the name BB rat (Chappel and Chappel 1983). There are two major inbred lines of BB rat designated the diabetes-prone (DP) BB/Wor rat and the diabetes-resistant (DR) BB/Wor rat, as well as several other BB sublines (most are not fully inbred) kept in laboratories throughout the world.

DIABETES-PRONE BB RAT. These nonobese animals spontaneously undergo abrupt onset of hyperglycemia followed by ketoacidosis. About 40-70% of these rats develop diabetes between 60 and 120 days of age. Affected animals die within 2 weeks unless exogenous insulin is given. BB rats with IDDM differ from humans in that they are severely lymphopenic, highly prone to infection, and predisposed to lymphopoietic malignancy (Like and Rossini, 1984). The lymphopenia of the BB rat affects all lymphocyte subsets to some degree, but of particular importance is a severe deficiency of $CD8^+$ cells and a complete absence of T-lymphocytes expressing the RT6 surface alloantigen (Greiner et al, 1986). This alloantigenic system is expressed on about 70% of $CD8^+$ and

50% of $CD4^+$ T-lymphocytes in the rat (Ely et al., 1983). In addition, Thyroiditis occurs in more than 50% of animals by 120 days of age (Sternathal et al., 1981).

DIABETES-RESISTANT BB RAT. In the Worcester colony, less than 1% of DR rats develop diabetes. They are frequently used as control animals. DR-BB/Wor rats are not lymphopenic and have normal numbers of T-lymphocytes that express the RT6 surface antigen (Butler et al., 1983). When an anti-RT6 antibody is injected into 30-day-old DR-BB/Wor rats, more than 50% of RT6-depleted rats become diabetes within 4 weeks (Greiner et al., 1987). These data suggest that a population of $RT6^+$ T-lymphocytes may play a key role in regulating the expression of autoimmune diabetes in BB rat.

At least three genetically determined factors (i.e., lymphopenia; an MHC association; and pancreatic lymphocytic infiltrate) have been found to associate with the development of diabetes in BB rats. The lymphopenia is inherited in an autosomal recessive pattern (Jackson et al., 1984; Herold et al., 1989). In addition to the phenotype of severe lymphopenia, a series of breeding studies indicate that the development of diabetes of BB rats is strongly linked to a gene within the major histocompatibility complex (MHC) (Colle et al., 1981; 1986a; Jackson et al., 1984; Buse et al., 1985). Colle and coworkers have utilized MHC recombinations and diabetes susceptibility maps to the class-II region of the BB rat (Colle et al., 1986b). It appears that all RT1 haplotypes which express class-II genes which are U are diabetogenic independent of class-I alleles. To date these studies in the BB rat are the strongest evidence that a class-II gene is essential for diabetes susceptibility

(for review see: Castaño and Eisenbarth 1990). Furthermore, Colle has also found a linkage of diabetes with acinar pancreatic lymphocytic infiltrates (Colle et al., 1986a).

Diabetes can be passively transferred to young BB rats (Koevary et al., 1983) or cyclophosphamide/irradiated histocompatible ($RT1^u$) non-BB rats (Koevary et al., 1985) with concanavalin A stimulated spleen cells from acutely diabetic donors. Prevention of disease has been achieved by T cell depletion or inactivation through neonatal thymectomy (Like et al., 1982) and anti-lymphocyte sera (Like et al , 1979). Islet-specific T cell lines or T cell hybridomas have also been isolated from the spleen and pancreas (Prud'homme et al., 1984; 1986). Moreover, it has been recently reported that $CD4^+$ T-cells alone are sufficient to transfer the disease in BB rat (Metroz-Dayer et al., 1990). Thus a T-lymphocyte-mediated immune response is a plausible explanation for development of insulitis and diabetes, but details of the destructive process remain to be determined. Since in BB rats cytotoxic $CD8^+$ T-cells are severely deficient in numbers and function (Prud'homme et al., 1988; Bellgrau and Lagarde, 1990), it is likely that diabetes is mediated by cooperation between T-helper ($CD4^+$), macrophages and natural killer (NK) cells.

The BB rat has been used widely as a model of human IDDM since its discovery. This has led to many insights into the immunopathogenesis of diabetes and to the introduction of immunomodulatory strategies of proven benefit, such as cyclosporine treatment (Laupacis et al., 1983).

B. NOD MOUSE

The Non-Obese Diabetic (NOD) is an inbred mouse strain. It was discovered by chance at the Shionogi Research Laboratories, Shionogi & Co., Ltd. in early 1974 and developed in the course of a breeding program to establish a cataract-prone subline (CTS) from non-inbred ICR mice (Makino et al., 1980). During the selective breeding, the non-obese diabetic mice were named the NOD strain and non-obese non-diabetic mice were named the NON strain. Like the BB rat, the NOD mouse is predisposed to develop a spontaneous form of diabetes and shares a number of important characteristics with human Type I diabetes. However, this disease is not accompanied by general immunodeficiency as in the BB rat. Thus, it is considered as one of the most suitable models for studying human Type I diabetes (Leiter et al., 1987).

Diabetes usually develops in NOD mice between the 12th and 30th wk of age, with an onset characterized by polydipsia, glycosuria, rapid weight loss, hyperglycemia and ketoacidosis (Table 1). The onset of hyperglycemia is preceded by insulitis, i.e., infiltration of the islets of Langerhans by mononuclear cells, mostly of T origin, which results in severe islet disorganization and in the death of insulin-producing β cell (Ohneda et al., 1984). Without insulin treatment the animals die within 4 to 8 weeks (Lampeter et al., 1989). These clinical and pathological features in the NOD mouse closely resemble human IDDM. Diabetes in the NOD mouse, unlike the human disease, includes simultaneous lymphocyte infiltration of salivary glands and other organs, and a female predominance (in some but not all NOD mouse colonies) (reviewed in: Tochino, 1987; Lampeter et al., 1989).

Table I

Comparison of clinical features at onset of diabetes in the human and the NOD mouse

	Type 1 (insulin-dependent) diabetes mellitus	
	Human	NOD mouse
Weight loss	Present	Present
Polydipsia	Present	Present
Polyuria	Present	Present
Hyperglycaemia	>15 mmol/l	20-30 mmol/l
Ketoacidosis	Common	Less severe
Serum insulin	Very low	Very low
Outcome without insulin	Lethal	Lethal
Sex preponderance	Female=male	Female≥male

Interestingly, the onset of diabetes in NOD mouse is heavily influenced by sex hormones, as indicated by the fact that NOD females are more prone to overt diabetes (70-80%) than males (20%) at 30 weeks of age although insulitis is present to a similar degree in both sexes. This difference in IDDM incidence disappears after castration (i.e., orchiectomy increases and ovariectomy reduces diabetes incidence) (Makino et al., 1981).

The genetic background of insulitis and overt diabetes has been investigated by backcross experiments with C57BL, NZB and NON mice (Prochazka et al., 1987). The results indicate that at least three recessive genes affect both the infiltration process per se and its severity, two of which are non MHC-linked. One gene controls the development of severe insulitis and appears to be incompletely dominant, and another is involved in the progression to diabetes, probably mediated by a lack of specific suppressor cells. The third is an MHC-linked gene that apparently influences the autoimmune response (Wicker et al., 1987). The linkage of diabetes to the MHC is interesting because the NOD mouse is I-E$^-$ and expresses a unique I-A class II molecule (I-A^{NOD}) (Acha-orbea and McDevitt, 1987; Nishimoto et al., 1987). The introduction of a transgenic class II molecule, I-E, protected NOD mice from insulitis and diabetes (Nishmoto et al., 1987). Furthermore, treatment with anti-I-A monoclonal antibody prevented diabetes in NOD mice (Boitard et al., 1988). Amino acid sequence comparisons suggest that aspartic acid at position 57 (Asp 57) of human HLA-DQ class II MHC and their murine homologues I-A molecules provide resistance to diabetes, whereas other amino acids are associated with susceptibility

(review in: Todd, 1987). Fitting this correlation, the single class II molecule of the NOD mouse ($I\text{-}A^{NOD}$) has serine at position 57 (Acha-Orbea & McDevitt, 1987). Using transgenic mice, however, three groups (Miyazaki et al., 1990; Slattery et al., 1990; Lund et al., 1990) have reported recently that Asp-57 does not necessarily prevent diabetes in NOD mice.

Another unique advantage of using NOD mouse as a model is that insulitis is in progress for a long period time before overt hyperglycemia. This prolonged and well defined prodromal period provides an excellent opportunity to test different approaches to immunotherapy early in the prediabetic stage and allows the autoimmune process to be halted before complete β-cell destruction and hyperglycemia have occurred.

III. AUTOIMMUNE FEATURES IN NOD MOUSE

The autoimmune nature of IDDM in the NOD mouse is supported by the presence of insulitis, the identification of anti-islet cell antibodies in the serum, the prevention of the disease by immunosuppression and most significantly, the adoptive transfer of disease with T cells.

III.1. INSULITIS

In NOD mice, insulitis is observed from at least the 4th week of age (Fujita et al., 1982). The major characteristic lesion is mononuclear cell infiltration around or into the pancreatic islets, including small lymphocytes with some macrophages, some plasma cells, and a very small number of neutrophils. The progression of insulitis is

not uniform within the same pancreas at any age, and lymphocyte infiltration specifically directed at the insulin-producing β cells is followed by selective degradation of the β cells, while A cells (with glucagon granules) and D cells (with somatostatin granules) are less affected. The typical symptoms of overt diabetes do not appear until most of the β cells (>90% β cell mass) have disappeared (Fujino-Kurihara et al., 1985).

Phenotyping T lymphocyte subsets involved in the insulitis has produced conflicting results (Kanazawa et al., 1984; Miyazaki et al., 1985; Signore et al., 1987; 1989). Some of those studies show $CD4^+$ cells (mainly helper/inducer) and MHC class-II positive cells as the most represented subsets while others show monocytes and B-lymphocytes are the predominant cell population. The reason of this disagreement is not yet clear. Within the T-lymphocyte population, $CD4^+$ cell are more frequently found than $CD8^+$ cells (mainly cytotoxic T lymphocytes).

These histological findings can be interpreted in two ways: the inflammatory mononuclear cells are "witnessing" the β cell destruction or alternatively are directly involved in the β cell damage. This latter hypothesis has been widely investigated by many researchers and much evidence suggests a direct damaging role of lymphocytes.

III.2. AUTOANTIBODIES

Human type I diabetes is characterized by the presence of several autoantibodies, namely islet cell cytoplasmic antibodies (ICA) detected on frozen sections of pancreas (Bottazzo et al., 1974), islet cell

surface antibodies (ICSA) (Lernmark et al., 1978) and insulin autoantibodies (IAA) (Palmer et al., 1983). Similar autoantibodies have also been found in NOD mice.

ISLET CELL SURFACE ANTIBODIES (ICSA): In NOD mice, ICSA appears in the prediabetic stage in 10 to 50% of the mice at 6 to 12 weeks of age and reaches maximal prevalence (50-70%) just before the onset of overt diabetes. After the onset, ICSA decreases. There are no sex differences in the prevalence of these antibodies (Kanazawa et al., 1984). Since ICSA appears at the age when insulitis is known to have already developed (insulitis begins at 4 5 weeks in NOD mouse), this suggests that these antibodies do not play a role in the initial islet damage.

ISLET CELL CYTOPLASMIC ANTIBODIES (ICA): Unlike the human counterpart, ICA reacting with frozen sections have not been reproducibly found in sera from NOD mice. However, Reddy and colleagues have recently detected these antibodies in NOD mouse sera using Bouin's fixed pancreas sections (Reddy et al., 1988).

INSULIN AUTOANTIBODIES (IAA): NOD mice also produce low levels of anti-"insulin" autoantibodies, and those mice with the highest levels are more likely to develop overt diabetes (Ziegler, 1989). Although much attention has been paid to the potential value of IAA as markers for active insulitis (that might ultimately proceed to diabetes), no study has yet emerged from which the predictive value of IAA can be judged (Wilkin, 1990).

OTHER AUTOANTIBODIES: Antibodies against the apical border of thyroid follicular cells and the salivary gland duct have been found in

NOD mouse model. In addition to organ-specific antibodies, antilymphocytic autoantibodies and antibodies against cell nuclei have also been detected both in diabetic patients and in animal models (Tochino, 1987). This indicates the presence of nonspecific polyclonal antibodies

The origin and significance of these humoral manifestations are still unclear in both human and animal models. Several reports have shown that sera from diabetic patients containing ICSA may alter a cell function and be cytotoxic to the a cell in the presence of complement (Kanatsuna et al., 1981). However, it is not proven that such antibodies can induce diabetes. In the NOD mouse model, one recent study indicated that these humoral anomalies are clearly disconnected from the occurrence of diabetes and even of insulitis (Lehuen et al., 1990). These investigators suggest that NOD mice, like other autoimmune strains, suffer from a genetically inherited defect of B lymphocyte regulation resulting in the hyperproduction of natural autoantibodies.

III.3. ADOPTIVE TRANSFER OF THE DISEASE

To investigate which cell subsets are involved in the a cell destruction, adoptive transfer of the disease has been done in both BB rat and NOD mouse. In the NOD mouse model, Wicker et al.(1986) first developed an adoptive transfer protocol that induces diabetes in NOD mice at an age when spontaneous diabetes is rarely observed. Their results showed that greater than 95% (79/82) of irradiated non-diabetic NOD recipients (at least 7 wks of age) became diabetic when adoptively transferred spleen cells were obtained from diabetic NOD mice or non-

diabetic NOD mice (more than 15 wks of age). Another study shows that diabetes can be adoptively transferred with T cells in newborn NOD recipients without irradiation (Bendelac et al., 1987). In these passive transfer studies, both male and female recipients are susceptible to disease in a dose-dependent manner. Several studies (Bendelac et al., 1987; Miller et al., 1988) suggest that both $CD4^+$ and $CD8^+$ T cell subsets are necessary to adoptively transfer diabetes. However, successful transfer of insulitis with only the T helper cells ($CD4^+$) has been reported in NOD mice (Wang et al., 1987; Charlton and Mandel 1988). Recently, transfer of insulitis with $CD4^+$ T cell clones (Bradley et al., 1990) has been reported. All these studies reveal that T-lymphocytes play a vital role in pancreatic a cells destruction.

III.4. PREVENTION OF THE DISEASE BY IMMUNOTHERAPY

A long prediabetic period with immunologic abnormalities and progressive β cell destruction gives a good chance to halt pancreatic β cell loss with immune intervention. Multiple interventions can prevent diabetes in NOD mouse model.

1) BONE MARROW TRANSPLANTATION: Bone marrow cells from NOD mice can transfer diabetes susceptibility to non-diabetes-prone F1 mice and to normal mouse strains (Yasumizu et al., 1987). In addition, normal bone marrow prevents diabetes when transplanted into NOD mice. This suggests the genes creating diabetes susceptibility act at the level of bone marrow precursor cells (Wicker et al., 1988; Serreze et al., 1988);

2) THYMECTOMY: Athymic nude mice with NOD background (Harada and

Makino, 1986) or NOD mice which have undergone neonatal thymectomy (Ogawa et al., 1985) do not develop insulitis and diabetes, suggesting a pivotal role for T-lymphoctes in the autoimmune process. However, thymectomy leads to a dramatic increase in the incidence of diabetes in NOD females when performed at weaning (3 wks) (Dardenne et al., 1989). This is possibly due to the loss of some T cell-dependent suppressor mechanisms by the thymectomy at this stage. Furthermore, thymectomy had no effect on NOD males and females when delayed beyond 6-7 weeks of age. These findings may reflect time dependent differences in the development of necessary lymphocyte subsets in the thymus (i.e., effector T cells first and T-suppressor cells last).

3) T-LYMPHOCYTE INJECTION: Injection of a non-islet cell reactive T lymphocyte line isolated from the islet of newly diabetic NOD mice into young nondiabetic NOD mice profoundly inhibited the development of diabetes and almost eliminated insulitis (Reich et al., 1989b). This result suggests that islets of recently diabetic NOD mice contain not only effector cells capable of damaging pancreatic a-cells (Reich et al., 1989a) but also cells that are able to suppress (or regulate) this autoimmune response. Development of IDDM may depend on the balance between these opposing forces.

4) MONOCLONAL ANTIBODIES TO T CELLS OR AGAINST CLASS-II MHC: Treatment with anti-Thy 1.2 mAb (T cells) can prevent diabetes but does not influence the progression of insulitis (Harada and Makino, 1986), whereas administration with L3T4 mAb (anti-CD4, detects mainly helper/inducer cells) abolishes both insulitis and diabetes (Koike et al., 1987; Wang et al., 1987; Shizuru et al., 1988). These results

indicate that the diabetes process in the NOD mouse is T-helper cell dependent. This point has also been indirectly proved by use of anti-class-II MHC antibody to prevent diabetes (Boitard et al., 1988), since this mAb is believed to block T-helper cells recognition of their antigen(s). In addition, Lyt-2^+ ($CD8^+$) cells (mainly cytotoxic lymphocytes) and macrophages are necessary for the development of insulitis since treatment with anti-Lyt-2 antibodies and silica particles has also been reported to prevent a-cell destruction (Charlton et al., 1988).

5) OTHERS: Cyclosporine and nicotinamide (an inhibitor of poly-ADP-ribose synthetase) have been used in the NOD mouse to reduce the incidence of insulitis and/or diabetes (Lampeter et al., 1989), and they also have some benefits in newly diagnosed Type 1 diabetic patients (Mendola et al., 1989; Pozzilli et al., 1989). Both agents have some immunomodulatory properties.

IV. T-CELL CLONING

IV.1. T-CELL CLONING TECHNOLOGY

It is possible to obtain cloned T cells that can be maintained in culture. This was made possible by the following technical advances: 1) The notion that phytohemagglutinin (PHA) was mitogenic for T lymphocytes (Nowell, 1960), 2) Mixed leukocyte cultures (MLCs) were developed to study immune responses to alloantigen (Bach and Hirschhorn, 1964), and the use of β-mercaptoethnal (2-ME) to improve lymphocyte responses *in vitro* (Cerottini et al., 1974), 3) T-cell growth factor (TCGF) was found

to be an essential ingredient for long-term growth of T lymphocytes (Gordon and MacLean, 1965; Kasakura and Lowenstein, 1965; Morgan et al., 1976). TCGF could be added from exogenous sources or could be produced endogenously by the activation of certain T lymphocytes. TCGF was renamed interleukin-2 (IL-2) a few years later (Aarden et al., 1979). IL-2 is thus the factor essential for growth of T cells in conditioned medium (CM), but other lymphokines such as interleukin-4 (IL-4, originally designated BSF-1 because of its activity on B cells) also can serve as a growth factor for some T cells (Paul and Ohara, 1987).

Most methods used to obtain cloned T cells depend on the presence of exogenous T cell growth factors (e.g., IL-2). Two rather distinct approaches have been used to derive and maintain human and murine T cell clones. The first employs Il-2 alone as the stimulus for T cell growth following T cell activation. However, this approach is frequently unsuccessful and such cells may rapidly develop phenotypic and karyotypic abnormalities. The second approach for deriving and maintaining T cell clones employs stimulating antigen and "filler cells" in addition to IL-2. In the case of cloned T cells reactive with conventional soluble antigens, the filler cells provide a source of Ia-positive antigen-presenting cells that are required for T cell stimulation (Kimoto and Fathman, 1980). Filler cells can be provided by spleen cells depleted of T lymphocytes (Lutz et al., 1981). The actual clones can be derived by limiting dilution (Glasebrook et al., 1981; MacDonald et al., 1980), culture in soft agar (Fathman and Hengartner, 1978), or micromanipulation (Zagury et al., 1975). Cloned T cells may be stored frozen in liquid nitrogen for several years.

The availability of T cell clones has greatly facilitated the analysis of the molecular basis for cellular interactions and for regulatory and effector functions of lymphocytes. But the major disadvantages of T cell clones are the requirements for continual restimulation with antigen or mitogen and careful monitoring of culture conditions to allow T cell lines to be maintained for long periods of time. In an attempt to overcome the major problem with long-term growth of T lymphocyte clones, T cell hybridoma techniques have been developed.

IV.2. T-CELL HYBRIDOMAS

The development of T cell hybridomas was a natural offshoot of the Köhler/Milstein method for making monoclonal antibodies (Köhler and Milstein, 1975). Following the first use of the thymoma BW5147 to immortalize mouse T cells by fusion, there were doubts about whether functional T hybrids could be obtained (Melchers, 1978). These soon disappeared when Taniguchi and Miller (1978) reported the first successful fusion between functional T cells and a T cell thymoma line. It appears that most important T cell functions can be immortalized by fusion with BW5147, including (with some expedients) cytotoxicity (Nabholz, 1980; Kaufmann, 1981).

T cell hybridomas have some advantages as well as a few drawbacks. Among the former are the ease with which they can be prepared and cloned, and their rapid growth to any desired number in the absence of accessory cells or growth factors (Beezley and Ruddle, 1982). One major drawback of hybridomas is that they are less stable than T cell clones, i.e., their function is more likely to disappear in a relatively short

time, unless rescued by regular recloning. Chromosome loss over the first few weeks after fusion is probably partly responsible, and thereafter many lines of interest have proved to be as stable as antibody-producing hybrids. Another problem concerns the extent to which the parent thymoma cell determine the properties of its hybrids.

The sought-after properties that make T cell hybridomas potentially so useful to immunology are, of course, their monoclonality and ability to grow rapidly and permanently The development of human-human hybridomas producing lymphokines is an important advance that may in the future be clinically useful in transplantation or allergy. Perhaps most impressive is the use of T cell hybridomas in elucidating the molecular nature and genetic origin of T cell receptor itself.

V. AUTOREACTIVE T-CELL STUDIES

V.1. T-CELL RECOGNITION OF ANTIGEN

T-cell recognition of antigen requires the formation of a multi-molecular complex that includes a class-I or class-II MHC molecule, a peptide antigen, and the T cell receptor (Schwartz, 1985). Helper T cells generally recognize antigen in association with class-II proteins, and most of the cytotoxic T cells recognize antigen in the context of class-I MHC products.

A. THE MAJOR HISTOCOMPATIBILITY COMPLEX (MHC)

Major histocompatibility complex (MHC) molecules are a group of cell surface proteins encoded by genetically linked loci on chromosome 6

in humans and chromosome 17 in mice. They play a major role in self vs. nonself discrimination and are intimately involved in immunological recognition. Class-I MHC products are present on most nucleated cells and are recognized together with nominal antigen by $CD8^+$ T cytotoxic cells. Class-II antigens are normally expressed on B lymphocytes, activated T cells, monocytes, macrophages, dendritic cells and thymic cortical epithelium. They are heterodimeric molecules that restrict the recognition by $CD4^+$ T cells of antigens on antigen-presenting cells.

Class-I MHC molecules in human include HLA-A, B, and C antigen. The homologous proteins in mice are H-2 K, D, and L antigens (Moeller, 1983). Class-II molecules in the human are HLA-DP, DQ, and DR antigens. There are two isotypic forms in mice, I-A and I-E. I-A is the homologue of HLA-DQ, and I-E is the homolog of HLA-DR (Kaufman et al., 1984).

B. T CELL RECEPTOR

The principal antigen-specific T cell receptor is a disulfide-linked heterodimeric transmembrane glycoprotein comprised of an α and a β chain (McIntyre and Allison, 1983, Allison and Lanier, 1987) and clonally distributed on T cells in association with the CD3 complex (Cleverrs et al., 1988). There is also a small subset (less than 5% of peripheral T cells) that expresses TCRs consisting of τ and δ subunits (Brenner et al., 1986). Like immunoglobulins, each α, β, τ and δ chain of the TCR contains a variable domain, involved in recognition, and a constant transmembrane "anchor" domain (Kronenberg et al., 1986). These polypeptides are encoded in the germline by various dispersed gene segments comprising variable (V), diversity (D, for the β chain gene),

joining (J), and constant (C) gene segments. Functional α and β genes are formed during T-cell development by DNA rearrangements that generate V-(D)-J genes, which are then joined to a C-region gene segment by RNA splicing (reviewed in: Kronenberg et al., 1986, Wilson et al., 1988). As a result of allelic exclusion, each T cell expresses only one TCR product. However, the potential T cell repertoire is extremely large. It is estimated that at least 10^7 unique TCR molecules can be created.

V.2. RESTRICTED TCR Vβ USAGE IN EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS (EAE)

EAE is currently the best characterized model of an antigen-specific, T cell-mediated autoimmune disease. It is an induced autoimmune disease of the central nervous system (Paterson, 1976) and mimics in many respects the human disease of multiple sclerosis (MS) (Lassman and Wisniewsky, 1979). The disease can be induced by immunization with the autoantigen myelin basic protein (MBP) (Fritz et al., 1985) or adoptive transfer of MBP-specific class II-restricted T cell clones (Zamvil et al., 1985). EAE is clearly mediated by $CD4^+$ T lymphocytes. Adoptive transfer was performed originally with lymph node cells (Paterson, 1960), T cells (Gonatas and Howard, 1974), selected T-cell subsets (Pettinelli and McFarlin, 1981), and later by isolation of encephalitogenic T-cell lines or clones (Ben-Nun et al., 1981; Zamvil et al., 1985).

The T cell receptors of T_H cells mediating EAE have been identified in two mouse strains of the H-2^u haplotype, B10.PL and PL/J (Urban et al., 1988; Acha-Orbea et al., 1988). Molecular

characterization of the TCRs used by B10.PL-derived T_H cells revealed the use of only two distinct Vβ gene segments, $V_\beta 8.2$ and $V_\beta 13$, and two distinct V_α gene segments, $V_\alpha 2.3$ and $V_\alpha 4.3$. The gene products of either V_β segments can pair with the gene products of either V_α segment to yield a total of four discrete types of T_H cells. The majority of T_H cells examined expressed the $V_\beta 8.2$ gene segment (84%) while the remainder expressed $V_\beta 13$ gene segment (16%). The distribution of V_α gene segments was less skewed among these MBP-specific T_H cells with 60% expressing $V_\alpha 2.3$ and 40% expressing $V_\alpha 4.3$. A similar restriction exists in the repertoire of PL/J mice recognizing this epitope, although differences exist in gene segment usage between the two strains. In an analysis of eight T_H clones mediating EAE in PL/J mice, Acta-Orbea and coworkers (1988) found that seven out of eight clones share the $V_\beta 8.2$ gene segment. Thus the $V_\beta 8.2$ gene segment is exactly the same as used by the majority of B10.PL and PL/J encephalitogenic T_H cells. Whereas the Vα gene segments are not the same, although their usage are also restricted. The TCR data from rats also showed that all the MBP(p68-88) specific T-cell hybridomas expressed the same V_β gene segment which shares 80% similarity with mouse $V_\beta 8.2$ (Burns et al., 1989). Such a high degree of restriction on the usage of V_β gene segments made it possible to prevent the disease by the administration of $V_\beta 8.2$-specific monoclonal antibodies. Furthermore, it has been recently reported that treatment with $V_{\beta 8.2}$ and $V_\beta 13$-specific antibodies in B10.PL mice led to a significant reduction in MBP responsiveness, a near-complete protection against EAE induction and a dramatic reversal of paralysis in affected animal (Zaller et al., 1990).

V.3. AUTOAGGRESSIVE T CELLS IN IDDM

A. ISOLATION OF ISLET-SPECIFIC T CELL CLONES IN RATS, MICE AND HUMANS

Passive transfer of diabetes with T-lymphocytes (both $CD4^+$ and $CD8^+$, or $CD4^+$ alone) from newly diagnosed BB rats and NOD mice, as mentioned earlier, has prompted the hypotheses that autoreactive T-lymphocytes mediate β cell destruction. Successful isolation of islet-specific T cells from BB rat and recently from NOD mouse and human provided a strong support to this hypothesis.

ISLET-SPECIFIC T CELLS IN BB RATS: Several T cell lines were isolated from the spleen and pancreas of newly diabetic BB rats, or BB x Buffalo hybrids (BBUF)(Prud'homme, 1984; 1985a; 1985b). These T cell lines proliferated and secreted IL-2 when challenged with islet cells or RIN-5F cells in the presence of $RT\text{-}1^u$ antigen-presenting cells. Most of these T cell lines had a helper phenotype ($W3/I3^+$, $W3/25^+$, $OX8^-$) and did not lyse islet cells as measured by chromium release, supporting their nature as T helper cells. To overcome the problem of long term maintenance of these T cell lines, T cell hybridomas with specificity for islet cell antigens have been produced in BB rats (Prud'homme et al., 1986). The response of both these hybridomas and T cell lines to islet-specific antigen was clearly MHC restricted (Prud'homme et al., 1987) and these results indicated that RT1 D class II antigen of the rat MHC (analog to human DR and mouse I-E) is the restricting element in islet cell recognition by these rat autoaggressive T helper cells.

ISLET-SPECIFIC T CELLS IN NOD MICE: A cloned islet-specific T cell line was derived from the spleen and LN of a newly diabetic NOD mouse (Haskins et al., 1988). It was a $CD4^+$ T lymphocyte that proliferated and made IL-2 in response to islet cell antigen and NOD antigen presenting cells. This clone could completely destroy islet graft tissue while similar grafts made with pituitary tissue were not affected. This suggested that the islet-specific T lymphocyte mediated islet destruction in a tissue-specific manner. Subsequently, a panel of islet-specific T cell clones from NOD mice was produced by the same group (Haskins et al., 1989). Most of their clones responded to islet-cell antigen from different mouse strains but only in the presence of antigen-presenting cells bearing the class II major histocompatibility complex of the NOD mouse. In vivo, the clones mediate the destruction of islet, but not pituitary, grafts. Furthermore, pancreatic sections from a disease transfer experiment with one of the clones showed a pronounced cellular infiltration and degranulation of islet in nondiabetic (CBA x NOD) F_1 recipients. Phenotypic analysis of these clones show that all of them are helper T cells. $CD8^+$ T cell clones have also been established together with T helper cell clones by another group (Reich et al., 1989a). They showed that a combination of $CD4^+$ and $CD8^+$ T cell clones was required to initiate diabetes in irradiated NOD and (NOD x BALB/c) F1 recipient mice. Interestingly, a more recent report by Haskins and McDuffie (1990) demonstrates that a islet-specific $CD4^+$ T cell c one was sufficient to initiate the disease process in young NOD mice.

ISLET-SPECIFIC T CELLS IN HUMAN: Human T cell clones with specificity for insulinoma cell antigens have recently been generated

from peripheral blood mononuclear cells of patients with newly diagnosed type I diabetes. Such clones proliferate in response to RINm 5F rat insulinoma membranes but not fibroblast membranes (Vliet et al., 1989). These insulinoma-specific proliferative responses are HLA-DR1 restricted. The insulinoma membranes are presented to the insulinoma-specific clones by HLA-DR, and not DQ molecules, which is a challenge to the hypothesis put forward recently by Todd et al., (1989). Furthermore, such cellular fractionation studies using rat insulinoma indicate that the antigenic determinant recognized by one of these clones is an integral membrane component of the insulin secretory granule (Roep et al., 1990). However, the pathogenic potential of these clones in unknown.

B. TCR Vβ USAGE IN NOD MICE

The reports of limited heterogeneity in TCR V_β elements in autoaggressive T cells of EAE have generated considerable interest. This encouraged people to investigate TCR V_β usage in islet-specific T cell clones isolated from diabetic NOD mice. So far TCR V_β usage analysis in NOD mice have generated apparently conflicting results. β-cell specific $CD4^+$ clones from spleens of diabetic NOD mice described by Haskins et al. (1989) appear to be heterogeneous in TCR V_β usage. Reich et al. (1989a) reported that all their $CD4^+$ and half of the $CD8^+$ clones isolated from the pancreas of recently diabetic NOD mice used $V_\beta 5$, known to be deleted during development in I-E expressing mice (Bill et al., 1988). A previous report showed that introduction of a transgenic class II molecule (I-E) protected NOD mice from insulitis and diabetes

(Nishimoto et al., 1987). Reich et al. (1989a) thus presumed that I-E molecules expressed in NOD transgenic mice delete $V_\beta 5$-bearing cells and consequently protect the animal from autoimmune β cell destruction. Unfortunately, this theory has not been confirmed by depleting the $V_\beta 5$ cells to protect NOD mice from diabetes. In addition, Lipes et al. (1989) reported insulitis in transgenic mice where almost all T cells express $V_\beta 8.2$; and Bacelj et al. (1989) showed that the injection of anti-$V_\beta 8$ prevents diabetes in cyclophosphamide-treated NOD mice. Moreover, Carnaud suggested that the T cell population was of $V_\beta 6$ phenotype since its depletion decreased the ability of splenocytes from diabetic NOD mice to passively transfer disease (see: Leiter et al., 1990). Therefore, the islet-specific TCR V_β expression in IDDM is still not well understood.

VI. RATIONALE

Insulin-dependent diabetes mellitus in human is a genetically programmed T cell-mediated autoimmune process that is directed against pancreatic β cells. Since the exact mechanisms of T cell actions in the immunopathogenesis of IDDM is not yet known, it would be valuable to find out which types of T cells are involved in the β cell destruction process and the manner of antigen recognition by those autoaggressive T cells. Furthermore, the knowledge of the TCR expressions of autoaggressive T cells might lead to immunological therapies with specific anti-V_β monoclonal antibodies. In this thesis the NOD mouse model was used mainly for its resemblance with human diabetes. Investigation of several aspects of autoaggressive T cell in autoimmune

diabetes was done through the construction of T cell hybridomas. The main reason for choosing T cell hybridomas instead of T cell clones was that they can be easily constructed, maintained and manipulated as discussed earlier.

PART II. MATERIALS AND METHODS

I. MATERIALS

I.1. ANIMALS

Diabetes-prone mice: NOD mice [H-2 K^d, I-A^{NOD}, I-E^-, D^b] were obtained from E. Leiter of Jackson Laboratory, Bar Harbour, Maine, and have been maintained in our animal facilities for several generations. These animals were tested weekly for urine glucose with Tes-Tape. Both males and females were used in the experiments. Samples were taken when they just became diabetic (usually 4-5 months old in our colony). Newborn NOD mice (7-10 days) were also used as source of islet antigens.

Non-diabetes-prone mice: mice of C3H/Hej [H-2 K^k, I-A^k, I-E^k, D^k] and C57bl/6 [H-2 K^b, I-A^b, I-E^-, D^b] were purchased from Harlan Sprague Dawley, Indianapolis, IN. Both newborn and adult mice are used as sources for different control islet antigens and antigen-presenting cells (APCs).

I.2. MEDIA AND REAGENTS

Complete medium: RPMI 1640 supplemented with 10% fetal calf serum (FCS), penicillin-streptomycin mixture (100 U/ml, 100 μg/ml respectively), 2 mM L-glutamine, 1 mM Sodium pyruvate, 10 mM Hepes, adjusted to pH 7.2 with 10N NaOH and filter sterilized. All the reagents were from Gibco Laboratories, Grand Island, NY., except the Hepes buffer which was from Boeringer Mannheim Canada.

T-cell medium: complete medium with 20% FCS, 20 mM Hepes and 50 μM 2-mercaptoethanol (2-ME) (Eastman Kodak Co., Rochester, NY.).

HAT supplement (10 mM hypoxanthine, 40 μM aminopterine and 1.6 mM thymidine) and HT supplement (HAT supplement minus aminopterine) were purchased from Gibco. Concanavalin A (Con A), collagenase, and 8-azaguanine used in this study were from Sigma Chemical Co., St. Louis, Mo.. DMSO was from Fisher Scientific; Polyethylene Glycol (PEG) was from BDH Medical Ltd, Poole, England; ^{3}H-thymidine was from Amersham, Oakville, Ont; Beta-Max liquid scintillation fluid was from ICN Biochemicals, Inc., Radiochemicals Division, Irvine, Ca.; Lympholyte-M was from Cedarlane Laboratories, Hornby, Ont.; and Percoll was from Pharmacia, Uppsala, Sweden.

I.3. CELL LINES AND ANTIBODIES

A. CELL LINES

CTLL-2: an interleukin-2 dependent mouse T cell clone, was obtained from the American Tissue Type Culture Collections, Rockville, Md.

BW 5147($\alpha^-\beta^-$): a subline of the mouse HAT sensitive thymoma BW5147 with a TCR α^-/β^- and CD3$^-$, CD4$^-$, CD8$^-$ phenotype, was a kind gift from Dr. Philippa Marrack

B. ANTIBODIES

List of antibodies used in this thesis				
Marker	Cell line Name	Source	Conjugate	Reference
CD3	145-2C-11	J. Bluestone	FITC*	Bluestone et al., 1987
CD4(L3T4)	GK1.5	ATCC (TIB 207)	PE*	Dialynas et al., 1983
CD8(Lyt-2)	3.155	ATCC (TIB211)	FITC	Sarminento et al., 1980
$V_{\beta 3}$	KJ-25-606 .7.5	P. Marrack	FITC	Pullen et al., 1988
$V_{\beta 6}$	44-22-1	H. Hengartner	FITC	Payne et al., 1988
$V_{\beta 8}$	F32.1	M. Bevan	FITC	Staerz et al., 1985
$V_{\beta 8.1/8.2}$	KJ16-133.18	P. Marrack	PE	Haskins et al.,1984
$V_{\beta 11}$	RR3-15	O. Kanagawa	FITC	Kao et al., 1988

*FITC represents fluorescein isothiocyanate
*PE represents phycoerythrin

II. METHODS

II.1. PREPARATION OF SPLEEN CELLS (ANTIGEN-PRESENTING CELLS)

Mice were killed and splenectomies were performed under aseptic conditions. The spleens were kept in ice cold Hanks balanced salt solution (HBSS) and then passed through a stainless steel tissue sieve

to make a cell suspension, which was washed twice with HBSS. Residual spleen fragments were removed by allowing the suspension to settle for 2 minutes. The supernatant was transferred to a centrifuge tube and spun at 1400 rpm in a bench top centrifuge for 10 minutes at room temperature. The cell pellet was resuspended in complete medium and irradiated at 2000R. They were used either immediately as APCs or frozen and stored in liquid nitrogen for use later.

II.2. PRODUCTION OF IL-2

Spleen cells were prepared from rats with a similar method as described above. They were suspended in T-cell medium supplemented with 5μg/ml ConA at a concentration of 3 x 10^6 cells/ml and cultured in either T-75 flask (50 ml suspension) or T-175 flask (150 ml suspension) for 24 hours. The culture was centrifuged at 400g for 20 minutes, the supernatant was collected and stored at -20°. This supernatant contains IL-2 and is called ConA supernatant or CAS. CAS was thawed just before use, first filtered with 0.8 μm pore filter and then sterilized with 0.22 μm filter.

II.3. ISLET CELL PREPARATION

Thirty baby mice pancreases were usually used for each preparation of islet cells. Pancreases were removed from baby mice (7-10 days old) and kept in cold HBSS. They were transferred to a petri dish containing 5 ml collagenase solution (2 mg/ml collagenase, 0.3 mg/ml aprotinin, 0.27 mg/ml DNase I, 1% BSA in HBSS), minced to about 1 mm pieces with curved scissors and transferred to a small beaker, the petri dish was

washed with an additional 25 ml of collagenase solution and also transferred to the beaker. Tissues were broken into smaller fragments by vigorous aspiration with a 5 ml plastic pipet for about 2 minutes, and then incubated at 37° for 10 minutes on a magnet stirring plate. The mixture was pipetted again for another 1-2 minutes and let sit for 3 minutes. The top 5 mls of the suspension was transferred to a 50 ml tube with 5 mls of HBSS and kept on ice. The digestion cycle was repeated twice (the incubation time for the last cycle was reduced to 3-6 minutes instead of 10 minutes) and all the digestion products combined together. All the above procedures were done under aseptic conditions. Islets of Langerhans were collected by centrifuging at 1400 rpm in a bench top centrifuge for 10 minutes and the supernatant was removed except the bottom 2 ml. The pellet was resuspended with a plastic pipet, carefully overlaid on the top of 5 ml percoll solution with a density of 1.045 mg/ml in HBSS and let sit for 5 minutes to allow the islet cells to sediment to the bottom of the tube. Percoll was then aspirated from the tube and the cell pellet resuspended in 10 ml of room temperature 0.29% trypsin solution. The tube was swirled gently in a 37° water bath for 10 minutes and a drop of the suspension was checked at 5 minutes under a microscope. When digestion was complete the suspension was washed three times with HBSS, the cells resuspended in 30 ml of complete medium and incubated at 37° overnight in a T-75 culture flask to allow cell surface antigens regenerate. After overnight incubation, the islet cells were irradiated at 2000 R and were than used immediately as antigens, or frozen and stored in liquid nitrogen.

II.4. ISOLATION OF T CELLS FROM THE PANCREASES OF DIABETIC NOD

MICE

One pancreas from a newly diabetic NOD mouse was used to isolate a cell suspension, using basically the same procedures as described above. 15 ml of collagenase solution were used. After digestion of islets with collagenase the mixture was washed three times with HBSS, the cells resuspended in 30 ml of T cell medium supplemented with 5 μg/ml Con A, 10^6/ml irradiated filler cells (NOD spleen cells), 10% CAS and cultured at 37° for 3 days. The cell culture was then washed every 3 days with HBSS and transferred to another flask to remove the fibroblasts which adhere to the flask. Nonadherent cells were recultured in fresh T cell medium supplemented with 10% CSA until T cell concentration reached about 10^6, when they could be used for cell fusions.

II.5. GENERATION OF T CELL HYBRIDOMAS

In this study, T cells were fused with a subline of the mouse HAT-sensitive thymoma BW5147 which is TCR α^-/β^-. BW5147 ($\alpha^-\beta^-$) cells were grown in complete medium supplemented with 20 μg/ml 8-azaguanine for one week to make them sensitive to HAT. Two days before fusion, thymoma cells were split every day and fed with fresh complete medium to induce a log growth phase. Prior to fusion, both T cell blasts and the BW5147 cells were washed twice with complete medium without FCS. They were then mixed at a ratio of 1:10 (T cell/BW5147 cell) and spun down in a 50 ml centrifuge tube at 1400 rpm for 10 minutes. The supernatant was removed from the tube and the pellet was dislodged from the bottom of the tube by gentle tapping. 1 ml of 35% PEG in RPMI was added to the tube with cells at a rate of 1 drop per second while rotating the tube slowly.

After two minutes, the cells were pelleted at 900 rpm for one and a half minutes in a bench top centrifuge. Two minutes later 8 ml of complete medium without FCS was added dropwise (within one minute) while rotating the tube, then 2 ml of FCS was added to stop the fusion process. The contents of the tube were mixed by gently tilting the tube back and forth and incubated in a sterile petri dish at 37° for one hour. The fusion product was diluted to the concentration of about 10^5 cells per ml with complete medium and cultured in 0.5 ml aliquots in 24 well plates at 37° for 24 hours. To each well, 0.5 ml of 2X HAT was added and the plate was incubated for another 10 days. At that time cells were fed with HT medium by aspirating 0.5 ml from each well and adding 1 ml of HT medium. This was repeated every 4 days by removing 1 ml from each well and adding 1 ml of fresh HT medium. In wells where hybridomas developed, the cells were transferred to 6 well plates and cultured for 5-6 days. At this point the hybridomas could either be tested or frozen with 30% DMSO and stored in liquid nitrogen.

II.6. SCREENING FOR ISLET-SPECIFIC HYBRIDOMAS

Screening for islet-specific hybridomas was done in 96 flat well plates. Hybridoma cells (10^4), APCs (5×10^5) and either 10^4 or 3×10^4 irradiated islet cells were co-cultured in triplicate wells with 250 μl of T cell medium. After 48 hours 100 μl of supernatant was collected from each triplicate well and combined in one tube. These supernatants were either tested for IL-2 immediately or frozen at -20° for later testing.

The IL-2 assay was performed as described by Gillis et al. (1978)

using the CTLL-2 cell line. This assay is based on the ability of IL-2 to stimulate the growth of CTLL-2 cells. CTLL-2 cells were grown in 30% CAS in T cell medium and fed every 3 days with fresh medium. At the day of testing, CTLL-2 cells were washed three times with HBSS before assay. 10^4 cells per well were cultured with various dilutions (1:16, 32 ... 1024) of ConA supernatant of spleen cells (CAS) (to establish a standard curve) or 200 μl of hybridoma supernatant (1:4 dilution) in T cell medium. After 20 hours, these cells were pulsed with 1 μCi [^{3}H] thymidine per well for 4 hours. Cells were collected with a cell harvester onto filter papers and [^{3}H] Thymidine uptake was measured, when the filter discs were completely dry, by liquid scintillation counting in 5 ml cocktail.

II.7. CELL SURFACE MARKERS AND TCR V_β ANALYSES

Flow cytometry analyses were performed with a fluorescence-activated cell sorter (FACS) analyzer (Becton-Dickinson Co., FACS division, Sunnyvale, CA) using the methods described by Loken and Stall (1982), and Braylan (1983) with some modifications. Single-color analyses were usually performed with FITC or PE conjugated antibodies. In some cases, the primary reagent was an unconjugated rat or mouse monoclonal antibody, and the secondary reagent was an affinity-purified FITC-conjugated anti-rat or anti-mouse IgG (Fc). In this study, the CD3 marker and TCR V_β's analyses were usually done with single-color analysis while the analyses of CD4 and CD8 markers were done with two-color techniques. For the two-color analyses, the anti-CD8 monoclonal antibody was conjugated with FITC, the anti-CD4 monoclonal antibody was

conjugated with PE.

Before staining dead cells were removed by centrifuging cultured cells through lympholyte-M at 1400 rpm for 20 minutes at room temperature. Live cells at the top of the tube were washed three times with HBSS and pelleted again. Primary antibodies were then incubated with the cells at 4° for 30 minutes; secondary antibodies were added to the cells after three washes with HBSS and incubated at 4° for another 30 minutes in the cases where two antibodies were used for staining. After washing with HBSS for three times all stained samples were fixed with 0.3 ml of paraformaldehyde before flow cytometry analysis.

Briefly, a mercury lamp in the FACS analyzer was used for excitation of both FITC and PE at 485±20 nm. Fluorescence emission was detected by selectively collecting 530±15 nm emission for FITC (green) and 575±25 nm emission for PE (red). The data were analyzed by a Consort 30 computer system (Becton-Dickinson, FACS Division). In determining the percentage of positive cells, a marker was set on the appropriate control histogram such that less than 0.5% of cells were to the right of this marker. By using this as a reference point, the percentage of cells in the specific antibody-stained sample histogram was calculated. For each histogram 5000 or more cells were analyzed.

TCR V_β expressions of the hybridomas were also analyzed through mRNA typing in collaboration with Dr. A. N. Theofilopulos (Scripps Clinic Research Foundation, La Jolla, California) using a multiprobe RNase protection assay with a full set of 17 available V_β probes as described in Singer et al. (1988). Briefly, V_β DNA inserts with a

distinct length for each V_β element were cloned into pGEM transcription vectors and ^{32}P labelled V_β-antisense RNA probes were synthesized by *in vitro* transcription in the presence of [α-^{32}P] UTP. These antisense RNA were used to hybridize with total cellular mRNA from the hybridomas in solution with excess amount of probes, and then the unhybridized RNA's were digested with RNase A and RNase T1. The protected probes were separated by polyacrylamide gel electrophoresis (PAGE) and the size of protected bands visualized by autoradiography.

PART III. RESULTS

I. T CELL HYBRIDOMAS FROM DIABETIC NOD MOUSE

The development of IDDM in human, mouse and rat is characterized by insulitis in which islet cells are specifically destroyed (Lampeter et al., 1989). The presence of islet-specific T cells in these pancreatic lesions has been documented in both rat (Prud'homme et al., 1984, 1986, 1987) and NOD mice (Appel et al., 1988; Reich et al., 1989a). To further study the islet-specific T cells, a panel of islet-reactive T cell hybridomas were produced from the pancreas of NOD mice and analyzed for their cell surface markers and TCR V_β expression.

I.1. CONSTRUCTION OF T CELL HYBRIDOMAS

Although hybridomas have been widely used in T cell studies and have proven to be important tools in the studies of organ-specific autoimmune diseases such as EAE (Burns et al., 1989) and IDDM in rat models (Prud'homme et al., 1986), to my knowledge no such T cell hybridomas have been constructed from the pancreas of diabetic NOD mice. It is our belief that these hybridomas are valuable research tools in understanding the immunopathogenesis of IDDM.

In this studies, a total of seven female and five male newly diabetic NOD mouse pancreases (1-7 days after diagnosis) were used as the source of T cells for 8 separate fusions. One or two pancreases from mice of same sex were used for each fusion experiment. The pancreases were digested in collagenase solution and whole digested pancreatic

tissue was cultured with T cell mitogen ConA 5μl/ml and filler cells 10^6/ml (i.e, irradiated NOD spleen cells) in T cell medium supplemented with IL-2 (10% CAS). After 5-7 days in culture, cell blasts (including mainly lymphocyte blasts and fibroblasts) could be observed under the microscope. Fibroblasts were removed by gently transferring the nonaherent cells to a fresh flask, since they tend to stick tightly to the wall of the culture flask. Lymphocyte blasts were recultured in fresh T cell medium supplemented with 10% CSA for 10-14 days. These blasts, mainly T cell blasts, were then fused with HAT sensitive mouse thymoma cells BW5147 ($\alpha^-\beta^-$) and seeded to several 24-well plates by limiting dilution with complete medium.

A total of 119 hybridomas were obtained from these eight fusions with varying yields ranging from 1 to 46 hybridomas per fusion. The results of the fusion experiments are summarized in Table II.

I.2. ANALYSES OF HYBRIDOMA CELL POPULATION

To assess the composition of this hybridoma panel, the cells were analyzed for their cell surface markers including CD3, CD4 and CD8 as described in materials and methods. The results are summarized in Table III. A large percentage of the hybrids (approx 46%) lack either CD3 or both CD4 and CD8. These hybrids would not be expected to be functional in our assay, and in fact none of these hybrids produced IL-2 when stimulated with islet cells and APC's (not show). The lack of these markers may be due to the loss of chromosomes carring corresponding genes following fusion, or in some cases to the fusion of non-T cells with BW5147. Some hybrids expressed both CD4 and CD8 (Table III). This

Table II

Generation of T cell hybridomas

Fusion No.	Sex	No. of pancrease	No. of hybridomas	Growth(%)*
1	F	1	4	1.9
2	M	1	1	1.0
3	M	1	8	7.4
4	M	1	13	10.0
5	M	2	16	11.1
6	F	2	1	2.1
7	F	2	46	26.8
8	F	2	30	11.1

* The hybridoma growth (%) was caculated by dividing the number of wells in which hybridomas grew with the total number of wells seeded.

may have resulted from the fusion of double positive ($CD4^+$ $CD8^+$) T cells, or from activation of the CD4 or CD8 gene following cell fusion, or from the growth of a $CD4^+$ and a $CD8^+$ clone in the same well (see below).

II. ISLET-SPECIFIC T CELL HYBRIDOMAS

II.1. FREQUENCY OF ISLET-REACTIVE HYBRIDOMAS

Fifteen to twenty hybridomas were tested for their islet reactivity in each experiment. Hybridomas were thawed, recultured for 3-7 days to get enough cells and examined under the microscope to make sure that they were healthy before testing. Triplicate wells with 10^4 hybridomas per well were co-cultured with 5×10^5 irradiated NOD spleen cells as antigen presenting cells (APCs) and 10^4 or 3×10^4 irradiated islet cells as the source of antigens as described in materials and methods. Hybridomas alone, NOD APCs alone, NOD islets alone and hybridomas plus NOD APCs were also cultured as controls. IL-2 is expected to be produced by the hybridoma cells only if they react with islet antigens or antigens on the APC's. Supernatant from each well was diluted four fold and tested for IL-2 with IL-2 dependent CTLL-2 cells. In this study, twelve of the 94 hybridomas tested (12.8%) were found to be reactive to islet antigens since high levels of IL-2 were produced with APCs and islet antigens (but not APC's alone), while the control wells produced only low levels of IL-2 (Figure 1). These positive hybridomas were tested 2-3 more times on different occasions within a period of about 1 year after initial identification to confirm the results and also to investigate the stability of these hybridomas. Most

Table III

Phenotype determination of hybridomas*

T cell markers	Percentage of total hybridomas in panel
$CD3^+CD4^+CD8^-$	23.4%
$CD3^+CD4^+CD8^+$	9.6%
$CD3^+CD4^-CD8^+$	21.3%
$CD3^-$	18.0%
$CD3^+CD4^-CD8^-$	27.7%

* These results were obtained by flow cytometry typing. T cell receptors were determined by single color staining with anti-CD3 monoclonal antibodies conjugated with FITC. CD4 and CD8 markers were determined by double color staining with anti-CD4 monoclonal antibodies conjugated with PE and anti-CD8 monoclonal antibodies conjugated with FITC.

Figure 1A. Response of T cell hybridomas

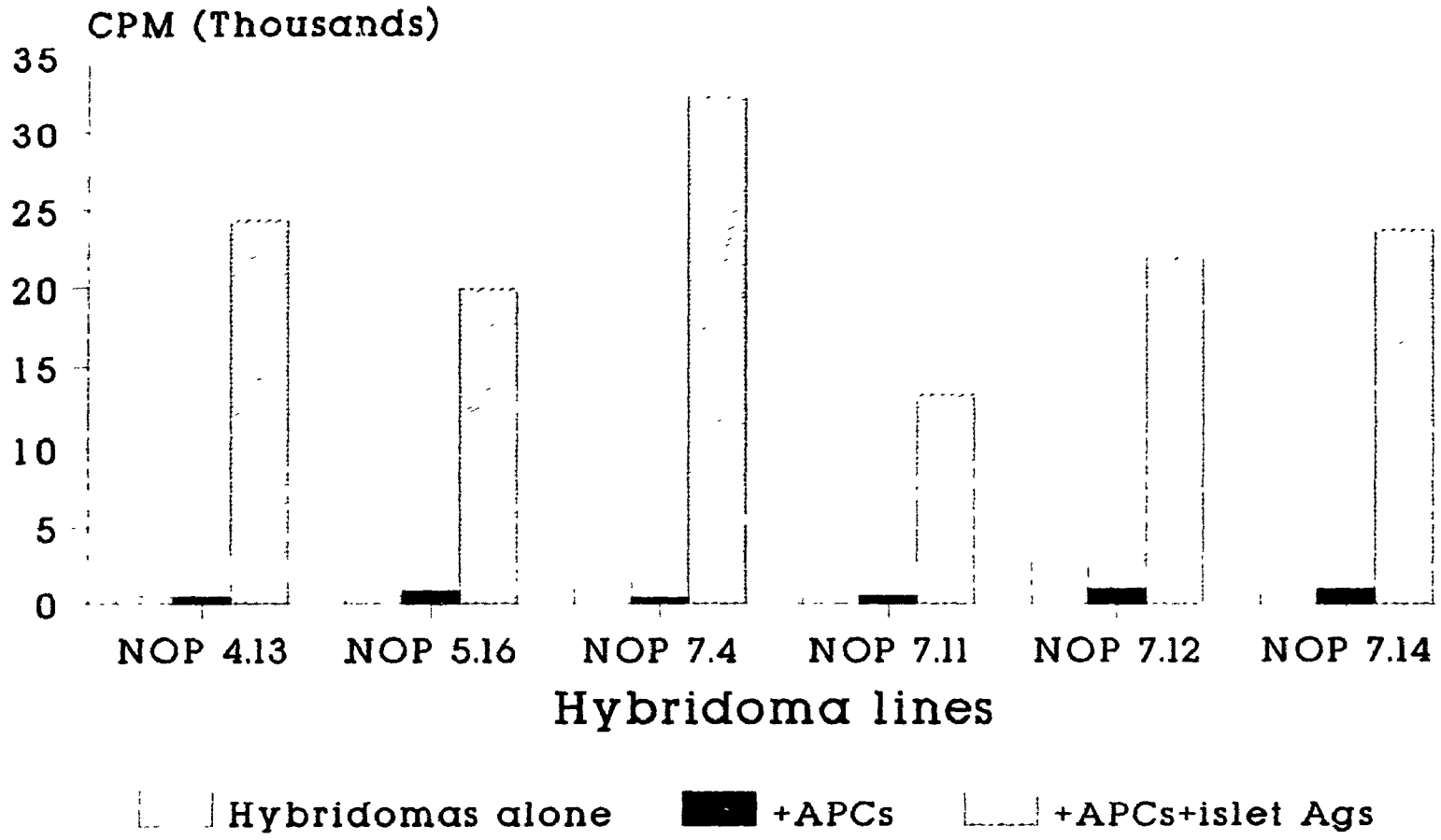
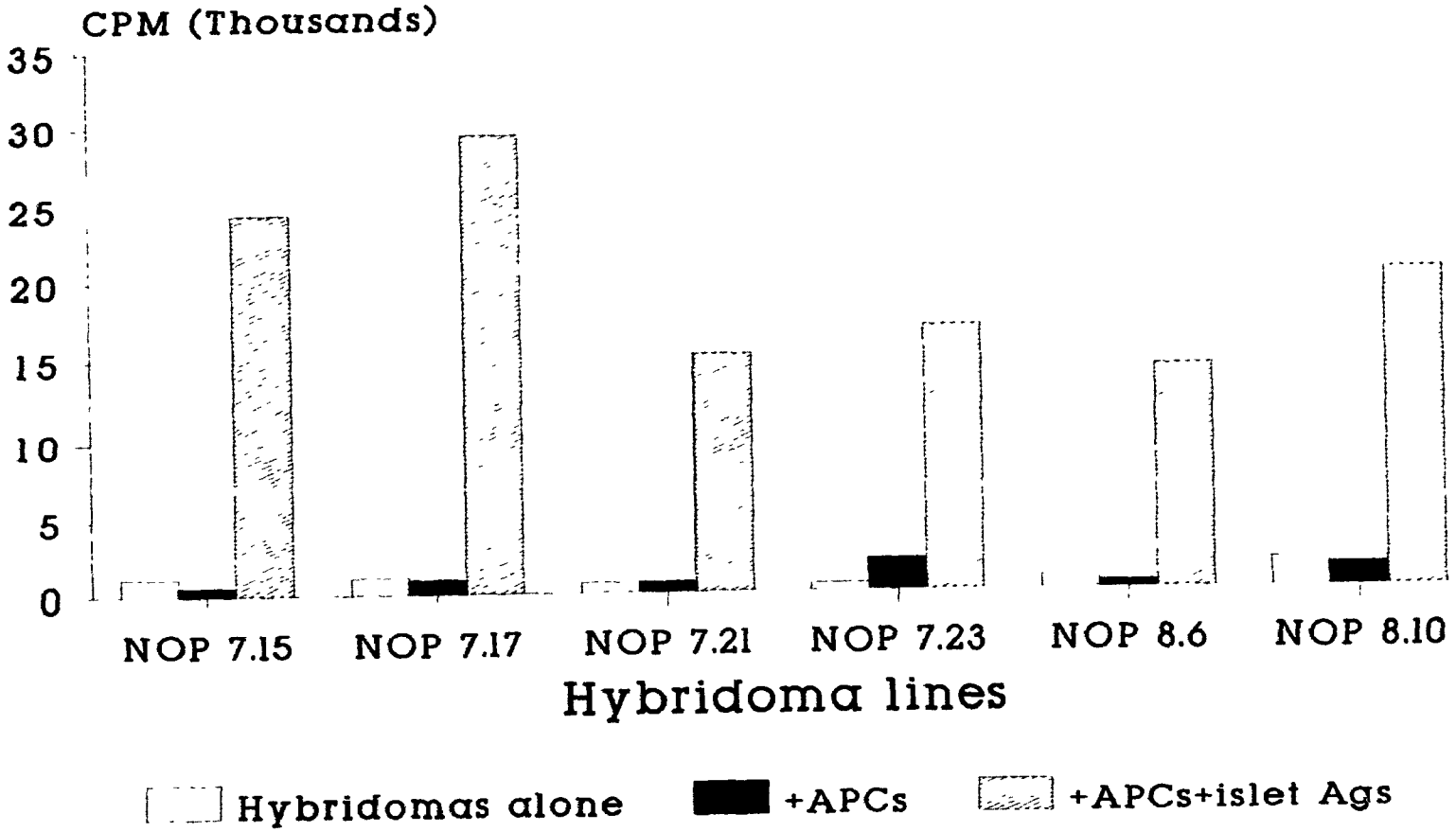


Figure 1B. Response of T cell hybridomas



of the hybridomas showed positive reactions in three tests while others showed positive reactions twice. Representative results are shown in Figure 1. Most of the hybridomas were relatively stable during the one year period although some hybridomas lost cell surface markers within a few weeks after the fusion. For example, some hybridomas (NOP 7.4, NOP 7.11, NOP 7.21) lost the CD4 marker and became unresponsive.

II.2. PHENOTYPE ANALYSES OF ISLET-REACTIVE HYBRIDOMAS

All the islet-reactive T cell hybridomas were analyzed for their cell surface markers including CD3, CD4 and CD8 with the same method as described above. The FACScan results are shown in Figure 2, Figure 3, and Figure 4. All of the islet-reactive T cell hybridomas were $CD3^+CD4^+CD8^-$ (Figure 3) except only one (NOP 4.13) which contained both a $CD4^+$ and a $CD8^+$ population (Figure 4). Note that BW5147 ($\alpha^-\beta^-$) cells were $CD3^-CD4^-CD8^-$ (Figure 2). TCR V_β expression analysis of NOP 4.13 indicated that hybridoma NOP 4.13 was not a clone since it had two different TCR V_β. This hybridoma was later subcloned through limited dilution (0.5 cell per well) with complete medium and cultured in 96-well plates for about two weeks. The cells were tested for islet-reactivity, subclone NOP 4.13.2 was found to be islet-reactive and all cells were $CD4^+$. This suggests that all of the islet-reactive T cell hybridomas were from fusions with T helper cells.

$CD4^+$ hybridomas comprise 33% of the total hybridoma panel (23.4% $CD4^+CD8^-$ plus 9.6% $CD4^+CD8^+$) (Table III). Therefore the frequency of islet-reactive hybridomas constitute almost 39% of all the $CD4^+$ hybridomas. This high frequency of islet-reactive T helper cell

FIGURES 2, 3, AND 4: PHENOTYPE DETERMINATION OF ISLET-REACTIVE HYBRIDOMAS.

The phenotype of each islet-reactive hybridomas (or the fusion partner cells, BW5147) was analyzed by flow cytometry typing for T cell surface makers CD3, CD4 and CD8. CD3 was identified by single color staining with anti-CD3 monoclonal antibodies directly conjugated with FITC as shown in Figures 2A, 3A, 4A. The horizontal scale represents the intensity of fluorescence from anti-CD3/FITC (in log scale), the vertical scale represents the number of cells.

CD4 and CD8 were identified by double color flow cytometry analysis using anti-CD4 monoclonal antibodies conjugated with PE and anti-CD8 monoclonal antibodies conjugated with FITC respectively as shown in Figures 2B, 3B, 4B. The horizontal scale represents the intensity of fluorescence from anti-CD8/FITC while the vertical scale represents the intensity of fluorescence from anti-CD4/PE, both of which are in log scale.

Figure 2. Flow cytometry typing of BW 5147 ($\alpha^-\beta^-$) cells as negative controls, showing that the fusion partner is $CD3^-CD4^-CD8^-$.

Figure 3. Flow cytometry typing of hybridoma NOP 7.15, representive of all the islet reactive hybridomas except one (Figure 4), showing $CD3^+CD4^+$ phenotype.

Figure 4. Flow cytometry typing of NOP 4.13. It is the only hybridoma which seems to be composed of a $CD3^+CD4^+$ population and a $CD3^+CD4^+CD8^+$ population. This suggests that it is not a clone.

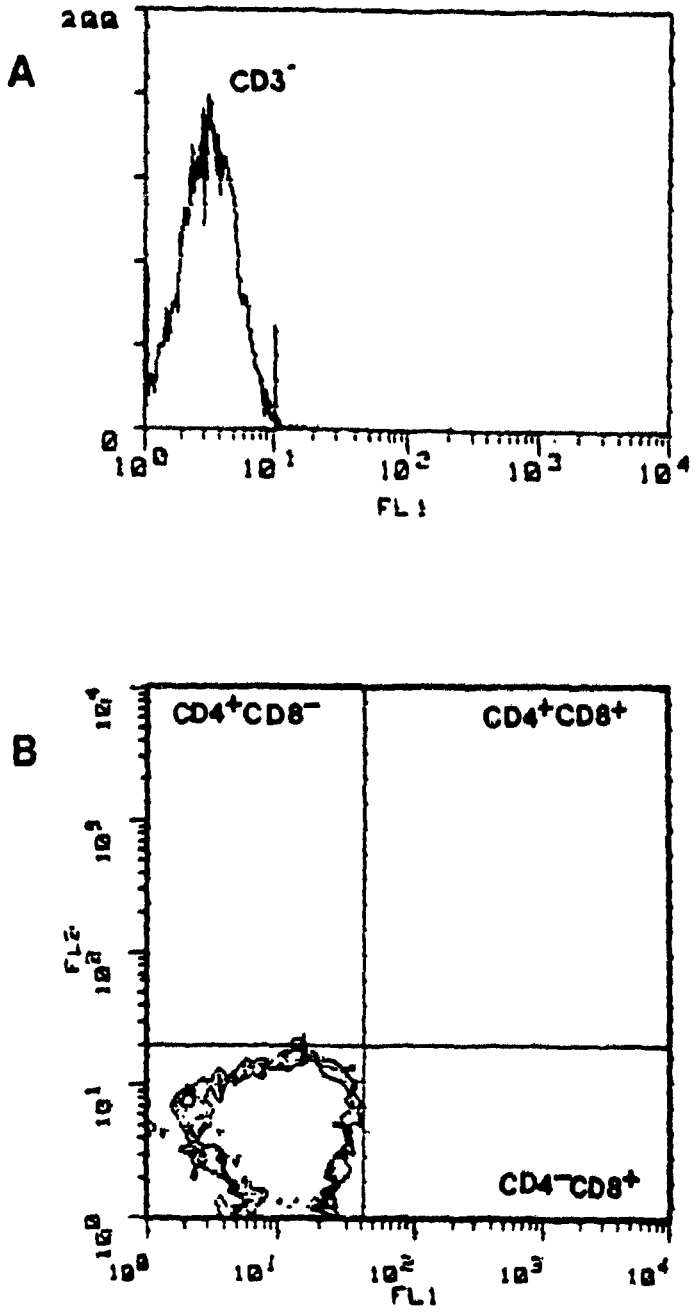


Figure 2. Flow cytometry typing of BW 5147 ($\alpha^-\beta^-$) cells

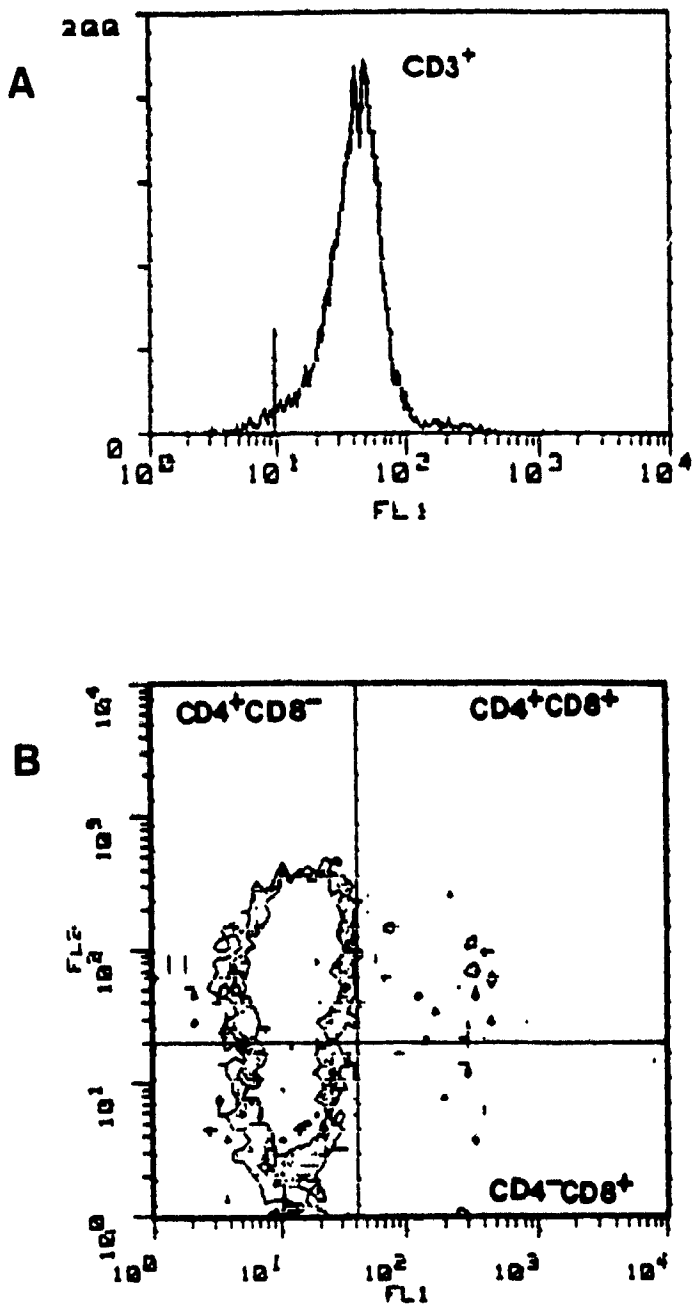


Figure 3. Flow cytometry typing of hybridoma NOP 7.15

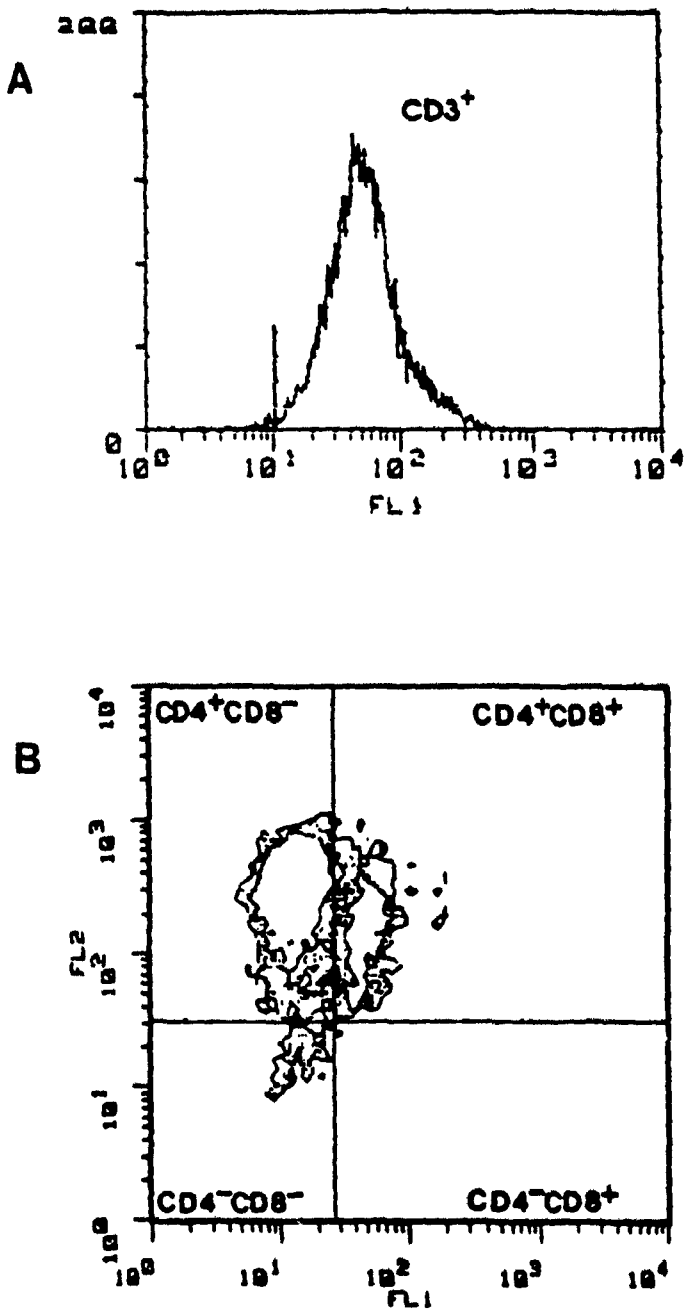


Figure 4. Flow cytometry typing of NOP 4.13

hybridomas suggests that even after the onset of overt diabetes there are still large numbers of potentially islet-reactive $CD4^+$ T cells in the pancreas of NOD mice.

II.3. ISLET-SPECIFICITY

To investigate if these islet-reactive T cell hybridomas react specificically with islet antigens in a MHC restricted manner, ten out of twelve these hybridomas were tested with different combinations of antigens and antigen presenting cells. Irradiated islet cells from C3H/Hej, C57BL/6 and NOD mice, NOD liver cells, rat thymus cells were used as antigens while irradiated spleen cells from C3H/Hej, C57BL/6 and NOD mice were used as APCs in the tests. These tests were done with the same procedure as described in section II.1 and the results are summarized in Table IV. All the islet-reactive T cell hybridomas reacted only with islet cells but not with liver cells or thymus cells (data not show). This indicated that they were islet-cell specific. Furthermore, it seems that all the reactions (except in three cases, see below) require the presence of APCs from NOD mice, which can not be substituted by APCs from other strains of mice. Some hybridomas responded to NOD islets, but not to islets from other strains, in the absence of NOD APCs. Possibly, our islet cell preparations contained macrophage/dendritic cells, which in some cases were sufficient to present islet-cell antigens and activate T cell hybridomas. Interestingly, some of the hybridomas did respond to islet cells from other strains (C3H/Hej, C57BL/6), but only in the presence of NOD APC's

Table IV.

Response of T hybrids to islet cells and APC's of NOD vs other strains

Hybrid name	NOD APC + Islet of			C3H/Hej APC + Islet of			C57BL/6 APC + Islet of			Without APC + Islet of		
	NOD	C3H	C57BL	NOD	C3H	C57BL	NOD	C3H	C57BL	NOD	C3H	C57BL
NOP 4.13	+	-	-	-	-	ND*	-	ND	-	-	-	-
NOP 7.11	+	-	-	-	-	-	-	-	-	-	-	-
NOP 7.12	+	+	+	ND	-	-	ND	-	-	+	-	-
NOP 7.14	+	-	-	-	-	-	-	-	-	-	-	-
NOP 7.15	+	-	-	ND	-	-	ND	-	-	+	-	-
NOP 7.17	+	+	+	-	-	-	-	-	-	-	-	-
NOP 7.21	+	+	+	ND	-	-	ND	-	-	+	-	-
NOP 7.23	+	+	+	-	-	-	-	-	-	-	-	-
NOP 8.6	+	-	-	ND	-	ND	ND	ND	-	-	-	-
NOP 8.10	+	-	+	ND	-	ND	ND	ND	-	-	-	-

*ND: not determined.

(but not with C3H/Hej, C57BL/6 APC's). These suggest that the NOD APC's process islet-cell antigens and impart MHC restriction to this response.

III. THE EXPRESSION OF TCR V_β ELEMENTS IN ISLET-SPECIFIC T CELL HYBRIDOMAS

It has been well documented in some other autoimmune diseases, especially experimental allergic encephalomyelitis (EAE) that the usage of TCR V_β repertoire was very limited and thus it has been possible to prevent EAE by injecting anti-TCR monoclonal antibodies in mice and rats (Urban et al., 1988; Acha-Orbea et al., 1988; Zaller et al., 1990). However, EAE is induced experimentally with dominant encephalitogenic peptides of myelin basic protein, such that it was not clear that a restricted expression of TCR V elements would also occur in a spontaneous autoimmune disease, i.e., IDDM in the NOD mouse.

TCR V_β expression of the islet-specific T cell hybridomas were analyzed in this study. This work was done by single-color flow cytometry analysis using available monoclonal antibodies against various V_β elements. The list of monoclonal antibodies available in our lab at the time of the experiments included anti-TCR $V_\beta 3$, $V_\beta 6$, $V_\beta 8$, $V_\beta 11$. The results of these experiments indicated that clones NOP 7.14 and NOP 7.23 expressed TCR $V_\beta 8$ (Figure 6 and Figure 7), clones NOP 7.15 and NOP 7.21 expressed TCR $V_\beta 3$ (Figure 8 and Figure 9), clones NOP 8.6 and NOP 8.10 expressed TCR $V_\beta 6$ (Table V) Several hybridomas were negative with all the anti-V_β antibodies. Through the collaboration with Dr. A Theofilopoulos at Scripps Clinic and Research Foundation, California, the TCR V_β expression of these islet-specific T cell hybridomas were

also analyzed through the analysis of TCR V_β transcription products by mRNA protection assay as described in the Methods section. These analyses confirmed the findings obtained through single-color flow cytometry. Combining both sets of data, TCR expression of these hybridomas were summarized in Table V. It is apparent that several V_β elements are represented with no obvious dominant type in these islet-specific T cell hybridomas.

FIGURES 5, 6 AND 7: TCR V_β TYPING WITH ANTI-$V_\beta 8$ MONOCLONAL ANTIBODIES.

Histogram A in each figure is the result from unstained cells as negative control. Histogram B's are the results of single color flow cytometry analysis with anti-CD3 monoclonal antibodies conjugated directly with FITC. Histogram C's are the results of staining with biotin-FITC (as control for two-step staining). Histogram D's are the results of staining with anti-$V_b 8$ monoclonal antibodies indirectly conjugated with FITC through biotin.

Figure 5. Staining of BW 5147 ($\alpha^-\beta^-$) as negative control. It is quite clear that the fusion partner cells are negative for all analyzed markers.

Figure 6. TCR typing of hybridoma NOP 7.14 showing that all the cells are $CD3^+$ (Figure 6B) while 75% of the cells are $V_\beta 8^+$ (Figure 6D). Islet reactive cells derived from NOP7.14 were almost 100% $V_\beta 8^+$ after subcloning (not shown).

Figure 7. TCR typing of hybridoma NOP 7.23 showing that all the cells are $CD3^+$ (Figure 7B) and more than 90% of the cells are $V_\beta 8^+$ (Figure 7D).

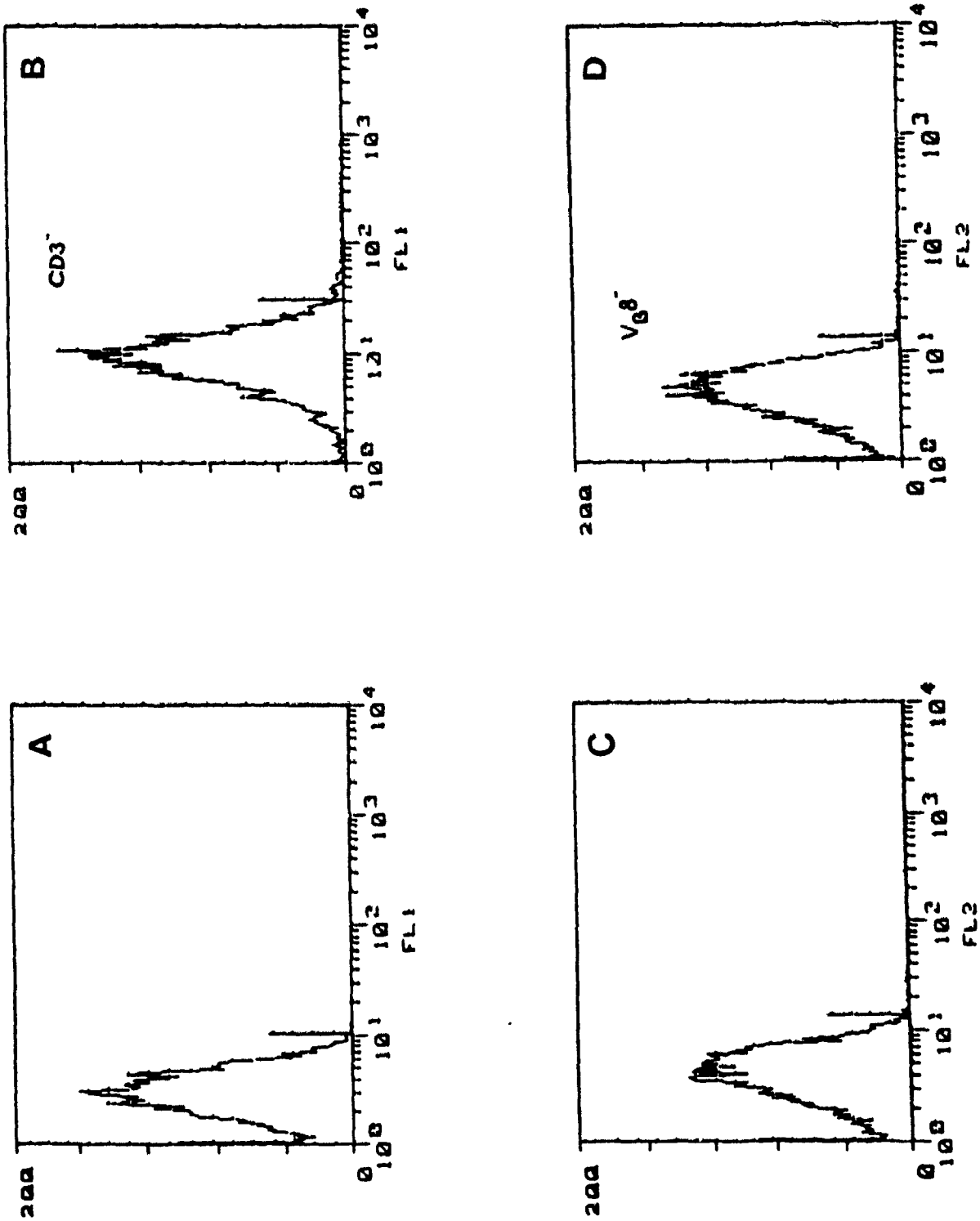


Figure 5. Staining of BW 5147 ($\alpha^-\beta^-$) as negative control

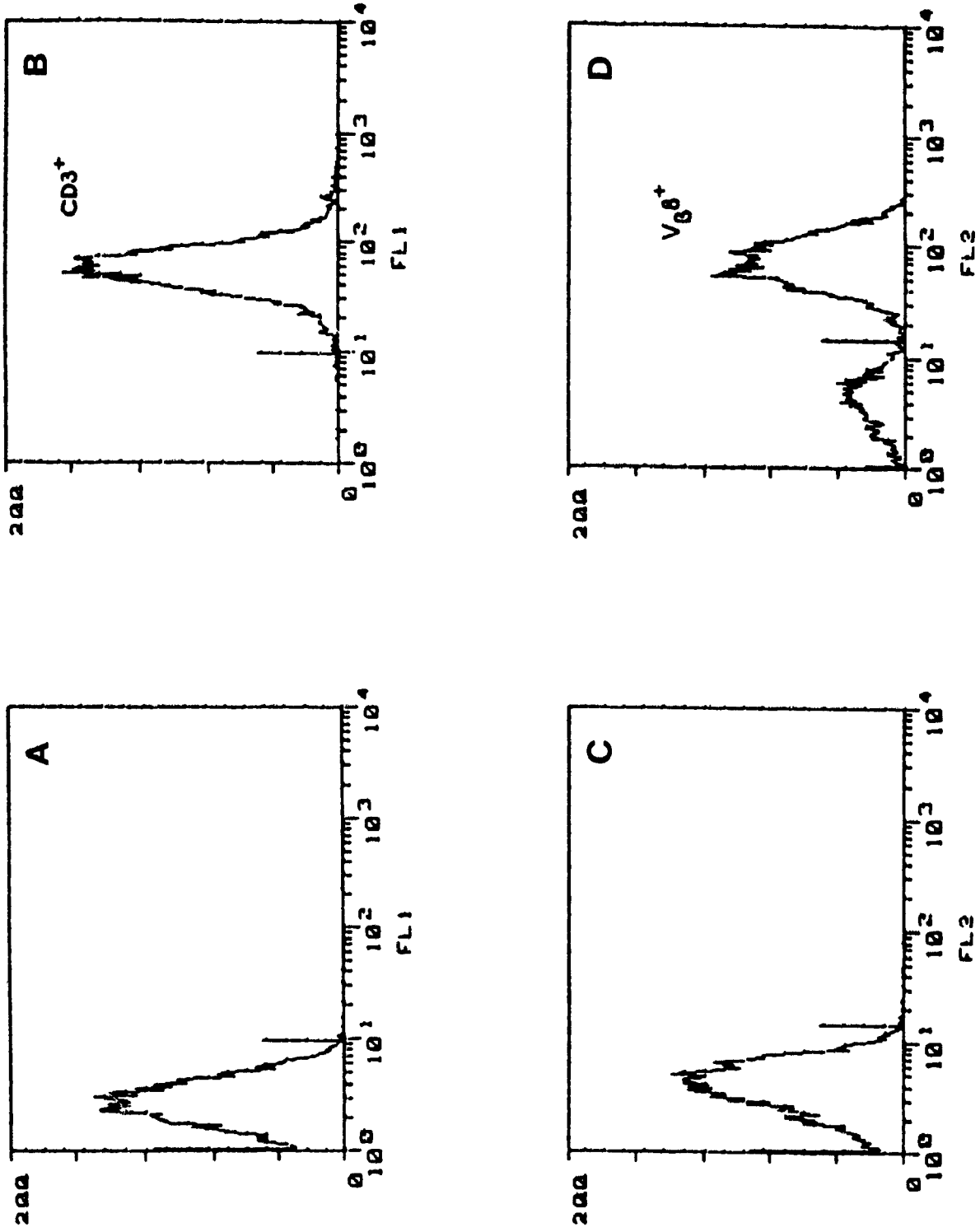


Figure 6. TCR typing of hybridoma NOP 7.14

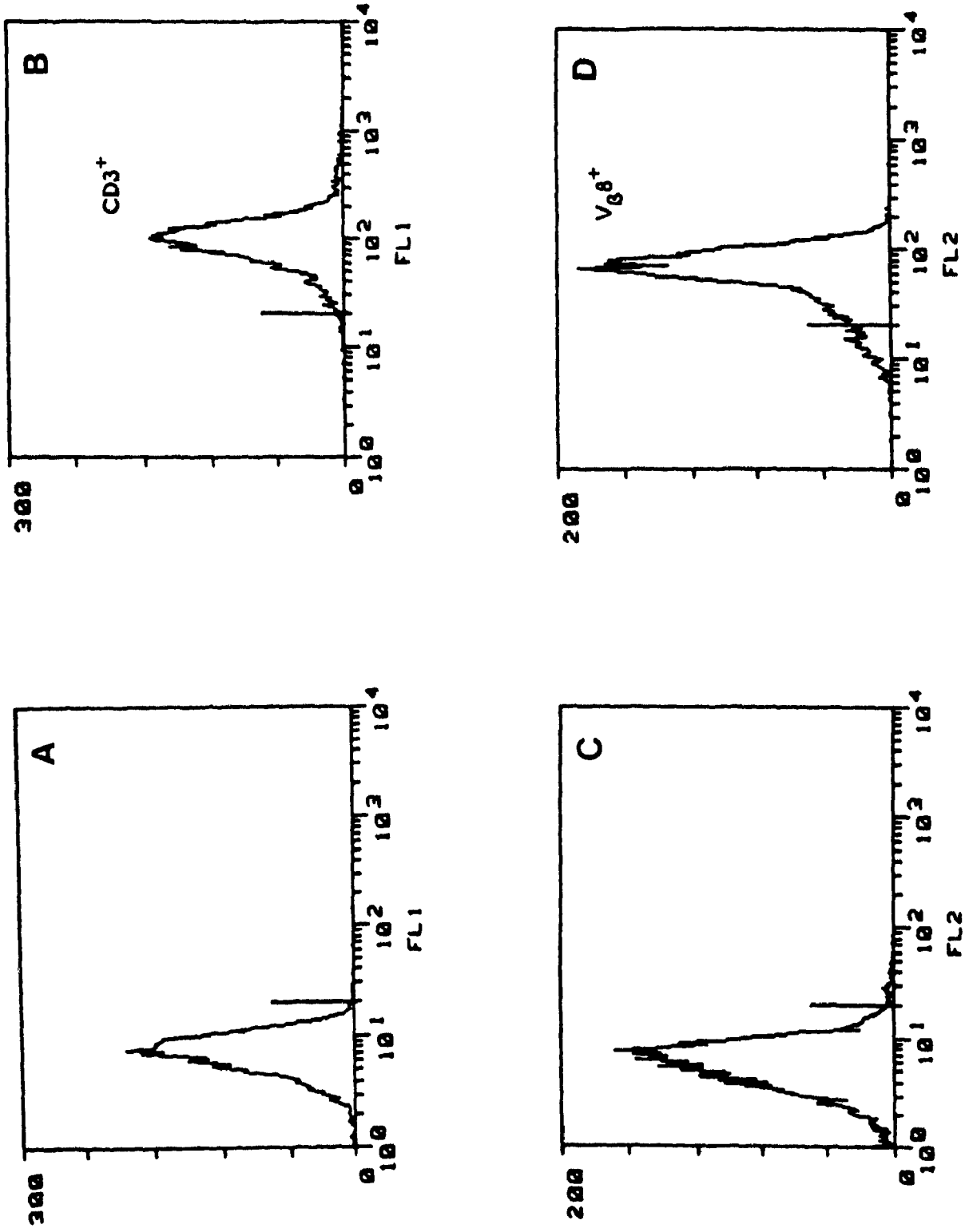
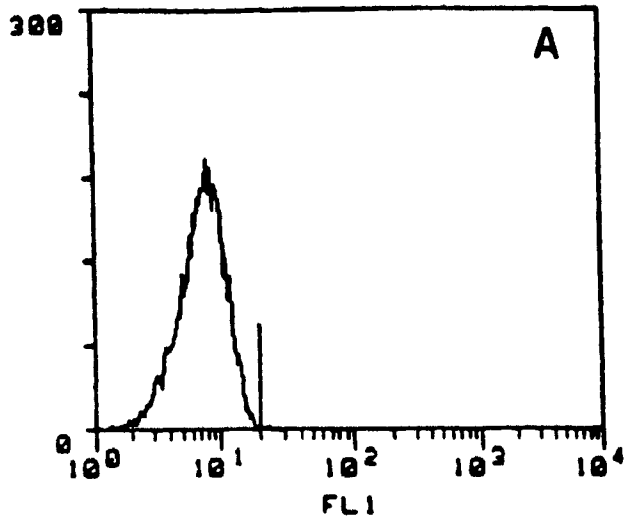
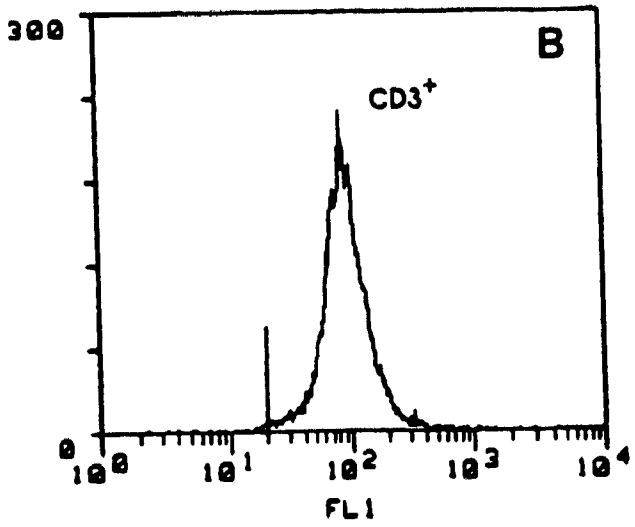


Figure 7. TCR typing of hybridoma NOP 7 23

FIGURES 8 AND 9: TCR V_β TYPING WITH ANTI-V_β3 MONOCLONAL ANTIBODIES.

Histogram A in each figure is the result from unstained cells as negative control. Histogram B's are the results of single color staining with anti-CD3 monoclonal antibodies conjugated directly with FITC. Histogram C's are the results of staining with anti-V_β3 monoclonal antibodies directly conjugated with FITC. Figure 8 represents the TCR typing of hybridoma NOP 7.15 and Figure 9 for NOP 7.21. In both cases, almost all of the cells are $V_\beta 3^+$.





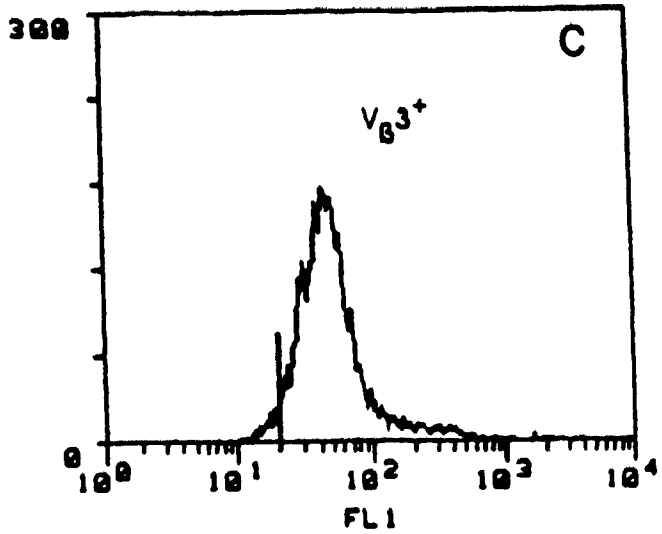
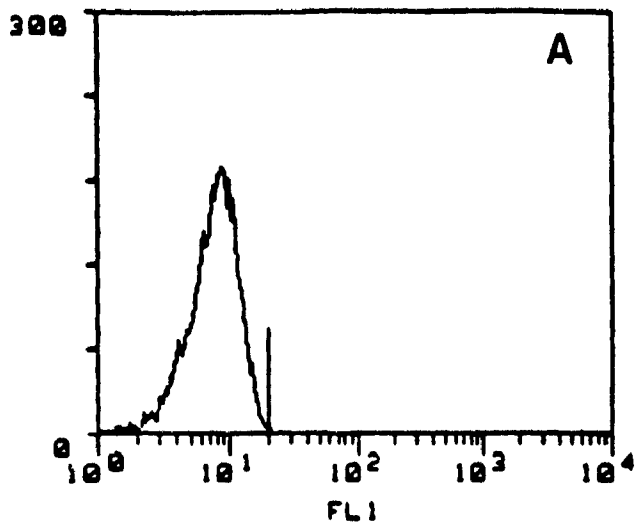
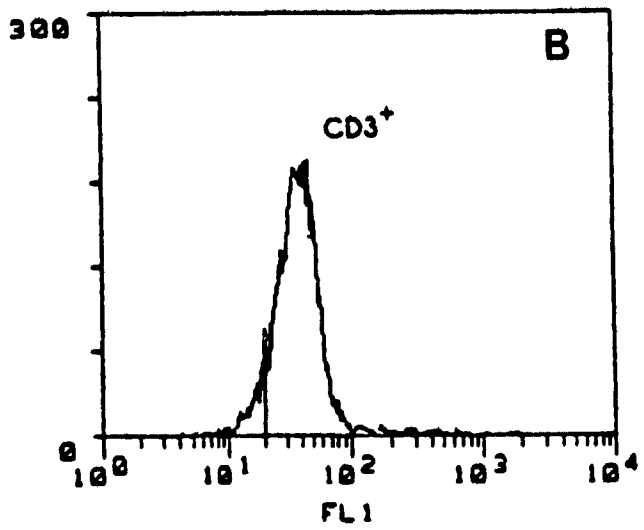


Figure 8. The TCR typing of hybridoma NOP 7.15





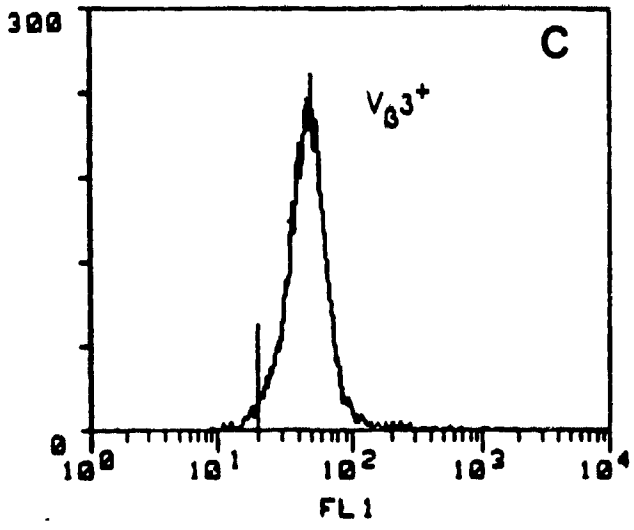


Figure 9. The TCR typing of hybridoma NOP 7.21

Table V

TCR V_β expression in islet-reactive T cell hybridomas

T cell Hybridoma	TCR V_β Expression*
NOP 4.13.2	$V_\beta 10$
NOP 7.12	$V_\beta 14$
NOP 7.14	$V_\beta 8.2$
NOP 7.15	$V_\beta 3$
NOP 7.17	$V_\beta 12$
NOP 7.21	$V_\beta 3$
NOP 7.23	$V_\beta 8.1$
NOP 8.6	$V_\beta 6$
NOP 8.10	$V_\beta 6$

* Typing by flow cytometry with anti-V_β monoclonal antibodies and/or in an RNase protection assay with specific mouse V_β probes (see test).

PART IV. DISCUSSION AND CONCLUSIONS

I. DISCUSSION

IDDM is now widely believed to be an chronic autoimmune disease in which activated T-lymphocyte destroy the insulin producing β-cells of the pancreatic islets. This view is strongly supported by isolating islet-reactive T-cells in BB rats (Prud'homme et al., 1984; 1986) and NOD mice (Haskins et al., 1989; Reich et al., 1989a). To better understand the exact mechanisms of T cell involvement in the immunopathogenesis of IDDM, a panel of islet-reactive T cell hybridomas have been constructed from the pancreas of newly diabetic NOD mice and their cell surface markers and TCR V_β expression have been characterized.

In this thesis the NOD mouse model was used mainly for its resemblance with human diabetes. Several aspects of autoaggressive T cells in autoimmune diabetes were studied though the construction of T cell hybridomas. Out of 119 hybridomas constructed, twelve were found to be islet-reactive (Figure 1) and all of them were $CD3^+CD4^+$ (Figure 3). This suggests that they most likely are derived from T-helper cells. Furthermore, a high percentage (39%) of the $CD4^+$ hybridomas were islet-reactive as demonstrated by analyzing the whole panel of hybridomas (Table 3). Moreover, these islet-reactive T hybridomas appear to specifically recognize islet cells in a MHC-restricted manner, since they only recognized NOD islet cells, or non-NOD islets in the presence of NOD APC's, but did not respond in the combined presence of non-NOD islet cells and non-NOD APC's (Table IV).

As mentioned above, all the islet-cell-specific hybridomas expressed the $CD3^+$ $CD4^+$ phenotype. This predominance of $CD4^+$ cells is probably related to the assay used to detect responses (IL-2 secretion), since autoaggressive $CD8^+$ T cells are also known to be present in NOD islets (Appel et al., 1988; Reich et al., 1989a). These NOD islet-cell-specific T hybridomas are remarkably similar to the BB rat-derived islet-cell specific T_H hybridomas previously described by Prud'homme et al. (1986, 1987). These results are also similar to those of Haskins et al. (1989) who produced NOD islet-cell-specific T_H cell clones from the spleen and lymph nodes of diabetic mice.

The percentage of $CD3^+$ $CD4^+$ hybrids which responded to islet cells in our panel was remarkably high, i.e., 39%. This may have occurred because of preferential activation of these T cells by ConA or more efficient fusion with mouse thymoma BW5147; however, we have no evidence that this is the case. We postulate that the high frequency of islet-cell-specific $CD4^+$ hybridomas reflects a high frequency of autoaggressive T cells in the islets of newly diabetic NOD mice. However, it is important to note that the frequency of islet-cell-reactive T-cell hybridomas varied widely from one fusion to another. It is likely that the number of autoaggressive T cells in islets fluctuates rapidly during the disease.

TCR V_β usage in NOD mice has also been analyzed by several other groups with apparently conflicting results. Interestingly, a previous report showed that the introduction of a transgenic class II molecule, I-E, protects NOD mice from insulitis (Nishimoto et al., 1987). It has also been documented (Kappler et al., 1987; Bill et al., 1988;

Theofilopoulos et al., 1989) that the expression of I-E results in intrathymic clonal deletion of T cells expressing $V_\beta 5$, $V_\beta 11$, $V_\beta 12$ and $V_\beta 16$ in some mouse strains (also $V_\beta 17$ but NOD mice cannot express that V_β segment at the gene level). However, I-E does not cause deletion of $V_\beta 5^+$ and $V_\beta 11^+$ T cells effectively in NOD mice expressing I-E transgenes (Böhme et al. 1990). NOD mice normally delete $V_\beta 3.1$ only partially, presumably because they are I-E$^-$, since when NOD mice are crossed with I-E$^+$ B10.BR mice (H-2^k, Mls-1^b, Mls-2^b) complete deletion is observed in the F1 progeny (Theofilopoulos, et al., 1989). From the analysis of TCR V_β deletions in crosses of NOD with other strains, NOD mice are believed to be Mls-1^b, Mls-2^a or Mls-3^a (Mls^c). Reich et al. (1989a) report preferential usage of $V_\beta 5$ in their NOD islet-reactive clones, a finding which was initially thought to be consistent with the view that I-E protects by inducing clonal deletion, but the hypothesis is refuted by the findings of Bohme et al (1990) in I-E$^+$ transgenic mice. Lipes et al. (1989) reported insulitis in transgenic mice where almost all T cells express $V_\beta 8.2$, and Bacelj et al. (1989) reported that the injection of anti-$V_\beta 8$ prevents diabetes in cyclophosphamide-treated NOD mice.

Analysis of our islet-reactive T hybridomas reveals expression of several V_β elements with no obvious dominant type. Out of 9 islet-reactive hybrids typed only $V_\beta 3$ and $V_\beta 6$ occur twice. However, this sample size is too small to determine whether or not some V_β elements will occur more frequently than others. Our findings with pancreas-derived T hybridomas appear to be similar to the findings of Haskins et al. (1989) who observed expression of several V_β elements in NOD islet-reactive clones obtained from lymph nodes and spleen. Furthermore, we

could not prevent IDDM by depleting $V_\beta 6$ or $V_\beta 11$ T cells in NOD mice (Prud'homme et al., 1990). In the latter study, depletion of $V_\beta 8$ had a statistically significant effect ($p < 0.02$), but 48% of treated mice still developed diabetes. Thus, $V_\beta 8$ cells appear to represent only one component of the islet-reactive T cell population. Taken together our results suggest that TCR V_β element usage is heterogeneous in NOD mice, unlike the situation observed in EAE. However, this view needs to be confirmed by studying greater numbers of islet-cell-reactive clones, particularly those that can be shown to be diabetogenic. Moreover, we have not yet studied the TCR $V\alpha$ chain in these islet-specific T cell hybridomas and our approach appears to reflect only the $CD4^+$ component of the anti-islet T cell response. It should also be stressed that the study of T cell hybridomas provides information on the spectrum of islet-reactive T cells, but does not indicate that these T cells are diabetogenic. It is possible, as suggested by Acha-Orbea et al. (1989), that one or a few T cell clone(s) starts a chain reaction and that at a later stage the autoimmune cells no longer reflect the original autoaggressive T cell population.

II. CONCLUSIONS

From the results presented in this thesis a few conclusions can be made. Together with other published research works these results indicate that autoaggressive T cells are involved in the immunopathogenesis of IDDM, and they might be responsible for the initiation of β cell destruction which eventually leads to overt diabetes. These autoaggressive T cells recognize islet antigens when

presented by self antigen presenting cells, i.e., the recognition of self-antigen is MHC restricted. TCR V_β usage by these autoaggressive T cells is not very restricted as compared to EAE. The reasons for this difference between autoimmune IDDM in the NOD mouse and EAE remain unknown. Possibly, in NOD mice T cells are responding against several islet cell antigens, or against multiple determinants on a large molecule. Alteratively, islet-cell-antigen recognition may not depend on TCR-V_β expression, but may be determined by other TCR elements (e.g., $V\alpha$, $J\alpha$, J_β).

The findings in this study lead us one step closer to the understanding of the immunopathogenesis of IDDM. Research of this nature would provide us with a frame in which to look for possible immunological interventions for IDDM. Hopefully that would lead to early diagnosis or treatments for IDDM.

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