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Persistent RNA virus infection and Development of Type I Diabetes

by

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ABSTRACT

Insulin-dependent diabetes mellitus (IDDM) or type I diabetes results from the specific destruction of insulin-producing pancreatic β-cells. Both genetic and environmental factors are thought to be involved in the pathogenesis of IDDM. Infection by viral agents such as encephalomyocarditis (EMC) virus in mice and coxsackie B virus (CB) are the pre-eminent environmental factors associated with IDDM. Other viruses such as retroviruses have made themselves conspicuous by their specific and constrained expression within the insulitis lesion of diabetes prone mouse strains.

The purpose of this study was to examine whether the persistence of viral genes either in the form of permanently integrated retroviruses or reverse transcribed viral RNA genomes in trans contributed to the pathogenesis of diabetes.

We were able to identify a unique endogenous retroviral sequence from the mouse insulinoma (MIN) 6N8a cell line which encoded for a sequence capable of eliciting humoral reactivity in non-obese diabetic (NOD) mice. We

also refuted the involvement of a candidate retroviral gene, IDDMK$_{1,2}$22, in the pathogenesis of IDDM.

cDNA persistence of both the D variant of EMC and CB serotype 4 was demonstrated in mice and a human respectively. Treatment with the antiretroviral agent AZT was shown to alter the progression of the diabetes in EMC-D infected diabetes susceptible mice.

It was concluded from these studies that retroviruses do not likely contribute directly to the pathogenesis of IDDM but may facilitate the persistence of other, diabetogenic RNA viruses via the action of endogenously encoded reverse transcriptase.

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LIST OF ABBREVIATIONS

ACH	Alberta Children's Hospital
AMV	avian myeloblastoma virus
APC	antigen presenting cell
ARC	animal resource centre
AZT	azidothymidine
β2m	β2 microglobulin
BB	biobreeding (rat)
BCIP	5-bromo-4-chloro-3-indoyl-1-phosphate
BSA	bovine serum albumin
CD	cluster of differentiation
CMV	cytomegalovirus
cNOS	constitutive nitric oxide synthase
CPH	carboxypeptidase H
E.coli	Escherichia coli
env	endogenous ecotropic murine leukemia virus envelope gene
GAD	glutamate decarboxylase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GP	glycoprotein

HERV	Human endogenous retrovirus
HGDMEM	high glucose Dulbecco's modified eagle medium
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
IAA	insulin autoantibodies
IAP	intracisternal type A particle
ICA	islet cell antibodies
ICSA	islet cell surface antibodies
IDDM	insulin dependent diabetes mellitus
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
IPTG	isopropyl-1-thio-β-D-galactoside
IVS	intervening sequence
kD	kilodalton
KRV	Kilham's rat virus
LB	Luria-Bertani
LCMV	Lymphocytic choriomenigitis virus
MHC	major histocompatability complex
MHV	mouse hepatitis virus

MIN6N8a	mouse insulinoma 6N8a
MMLV	Moloney murine leukemia virus
MMTV	mouse mammary tumour virus
NBT	nitroblue tetrazolium
NIDDM	non-insulin dependent diabetes mellitus
NK	natural killer cell
NO	nitric oxide
NOD	non-obese diabetic (mouse)
NON	non-obese non-diabetic (mouse)
NOS	nitric oxide synthase
ORF	open reading frame
p2C	protein 2C (coxsackievirus)
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethlyene glycol
p.i.	post infection
RT	reverse transcriptase
SIV	simian immunodeficiency virus
SLE	systemic lupus erythematosus

SPF	specific pathogen free
Taq	thermus aquaticus
TBEV	tick born encencephalitis virus
Th	helper T-cell
TLC	thin layer chromatography
TM	transmembrane
TNF-α	tumour necrosis factor α

1. Introduction

Diabetes is defined as any of a group of metabolic disorders characterized by excessive thirst and the production of abnormal amounts urine (Table 1.1, Table 1.2). The most common form is diabetes mellitus, which involves the improper metabolism of sugar and starch. In its multitude of forms diabetes affects approximately 5% of Western populations (Notkins, 1979). Amidst this multitude, insulin dependent diabetes mellitus (IDDM) and the characteristically distinct non-insulin dependent diabetes mellitus (NIDDM) account for >90% of afflicted individuals (Table 2). It is the insulin dependent form of diabetes mellitus and specifically its viral etiology, which are the concerns of this dissertation.

Historically, diabetes was among the earliest of documented diseases. An Egyptian papyrus c.1500 BC describes an illness linked with the passage of copious amounts of urine. It was not until the 2nd century AD, however, that the term 'diabetes,' i.e. to pass through, was coined by the Greek physician Aretaeus. It had been known for centuries that diabetic urine was sweet to the taste. In the 18^{th} century Matthew Dobson showed that the sweetness in diabetic urine was indeed due to sugar. It was

Table 1.1* The WHO classification of diabetes mellitus and allied categories of glucose intolerance.

A Clinical Classes	
Diabetes mellitus:	Insulin dependent diabetes mellitus
	Non-Insulin dependent diabetes mellitus (a) Non-obese (b) Obese
	Malnutrition-related diabetes mellitus
	Other types of diabetes mellitus: (associated with specific conditions and syndromes)
	Gestational diabetes mellitus
Impaired glucose intolerance:	Non-obese
	Obese
	Associated with certain conditions and syndromes
B Statistical classes	
Previous abnormality of glucose intolerance	
Potential abnormality of glucose intolerance	

* Adapted from Keen H, 1997

Table 1.2* Other types of diabetes

Diabetes due to pancreatic disease:
Chronic or recurrent pancreatitis
Haemochromatosis
Diabetes due to other endocrine disease:
Cushing's syndrome
Acromegaly
Thyrotoxicosis
Phaeochromocytoma
Hyperaldosteronism
Glucagonoma
Diabetes due to drugs and chemicals:
Glucocorticoids and corticotrophin
Diuretics
β-blockers
β_2 -antagonists
Phenytoin
Cyclosporin
Nicotinic acid
Diazoxide
Vacor (rodenticide)
Diabetes due to abnormalities of insulin or its receptor:
Insulinopathies
Receptor defects
Circulating antireceptor antibodies
Diabetes associated with genetic syndromes:
DIDMOAD syndrome
Myotonic dystrophy and other muscle disorders
Lipoatrophic diabetes
Type 1 glycogen storage disease
Cystic fibrosis

* Adapted from Keen H, 1997

Table 2* IDDM vs. NIDDM

	Insulin-dependent (IDDM ; Type 1)	Non insulin-dependent (NIDDM ; Type 2)
Age at onset:	Usually under 25	Usually over 40
Prevalence:	0.2-0.5%	2-4%
Sex distribution:	Male = Female	Female preponderance
Clinical Onset:	Acute to subacute; ketosis may be present	Chronic nonspecific symptoms
Insulitis:	Present	Absent
Peripheral lymphocyte abnormalities:	Present	Absent
Association with other autoimmune diseases:	Yes	No
Genetics:	Frequently Familial; strong association with histocompatibility markers HLA-Dr 3/4	Very frequently familial; no association with histocompatibility markers
Insulin treatment:	Always necessary	Not always necessary; patients may respond to diet and/or oral agents
Former terminology:	Juvenile onset diabetes Ketosis-prone diabetes Brittle diabetes	Adult onset diabetes Ketosis-resistant diabetes Maturity onset diabetes of the young(MODY)

* Adapted from Rossini AA, 1985.

not long after that John Rollo added the adjective 'mellitus,' i.e. honey, to the descriptive nomenclature of the disease.

Within the past two hundred years the defect in acute diabetics has been determined to reside within the pancreas, specifically the Islets of Langerhans. This sub-tissue structure was first identified by Brockman in the early 19th century in fish but the islets bear the name of the man who identified them in mammals in 1869. Relevance of the pancreas to diabetes was established by von Mering and Minkowski in the late 19th century via the demonstration that surgical removal of the pancreas in the dog led to clinical diabetes. The observation was later made by Opie that, in humans affected with the disease, the pancreatic islets appeared to deviate from their typical structure.

The seminal work in modern diabetes research took place in the early part of the 20th century. In 1921 the team of Frederick Grant Banting, Charles Herbert Best, James Bertram Collip, and John James Rickets MacLeod at the University of Toronto prepared active pancreatic extracts with the capability of lowering elevated blood glucose levels in dogs. Within months the active component in this

extract, insulin, was being administered with complete efficacy in children afflicted with IDDM.

IDDM, also known as Type I diabetes, is the result of the destruction of insulin producing pancreatic β-cells. The evidence is considerable that this destruction is the result of autoimmune activity during a prolonged, subclinical prediabetic period (Bach, 1988; Castano and Eisenbarth, 1990; Honeyman and Harrison, 1993; Rossini et al., 1993). Histological analysis of pancreata from human IDDM patients revealed mononuclear cell infiltration of the islets of Langerhans (Gepts and Lecompte, 1981b). It was later determined that the infiltrating cells were composed of T and B lymphocytes, monocytes/macrophages, and natural killer (NK) cells (Bottazzo et al., 1985; Hanninen et al., 1992b; Itoh et al., 1993b). Islet-reactive autoantibodies (Baekkeskov et al., 1990b; Kolb et al., 1988b; Palmer et al., 1983b) and islet-specific T-cells (Roep, 1996) have been found in IDDM patients, an indication that islet destruction follows an autoimmune pathway. At present a large body of experimental evidence suggests a critical role for T-cell mediated autoimmunity in animal models of IDDM (Haskins and Wegmann, 1996; Koevary et al., 1983;

Koike et al., 1987; Nagata et al., 1989; Ogawa et al., 1985b; Shizuru et al., 1988; Wicker et al., 1986b).

1.1. Genetics of IDDM

The hereditary nature of IDDM has long been accepted based on the high rate of familial transmission. In Caucasian populations the risk of IDDM is approximately 0.4%. In siblings and children of diabetics that selfsame risk rises to 7% and 6% respectively (Rotter et al., 1990). The concordance rate in monozygotic twins is 36% (Olmos et al., 1988; Risch, 1987). The high discordance rate in these twin studies indicates that susceptibility genes have low penetrance, i.e. the majority of individuals with genetic predisposition for IDDM will not develop the disease. Thus the greater portion of disease susceptibility, perhaps as much as 70%, must be accounted for by environmental factors.

The first evidence implicating human leukocyte antigens (HLA) was found in 1973 with an association being established with the Class I molecule HLA-B15 (Singal and Blajchman, 1973). It soon became clear, however, that a much stronger association existed with Class II antigens.

Greater than 95% of IDDM patients were found to carry the HLA-DR3 or DR4 antigens compared with 50% of the non-diabetic population (Svejgaard and Ryder, 1989). Fine mapping of susceptibility genes revealed that diabetogenic haplotypes carry a DQB1 allele encoding an uncharged amino acid, i.e. alanine, serine, or valine, at position 57 of the HLA β-chain. Non-diabetogenic haplotypes encoded for the charged amino acid, aspartic acid, in the same position (Horn et al., 1988; Todd et al., 1987) (Table 3).

1.2. The autoimmune response against pancreatic β-cells

IDDM is the direct result of an autoimmune attack against insulin-producing pancreatic β-cells. Evidence exists supporting the activity of both humoral and cell-mediated immunity. It is well established that cell-mediated immunity plays a critical role in the pathogenesis of IDDM. Humoral immunity is evidenced by the presence of a variety of autoantibodies that are not present in the non-diabetic population.

Table 3* Diabetes associated HLA polymorphisms

Gene encoding α-chain	Gene encoding β-chain	Corresponding HLA antigen	Effect on diabetes susceptibility
DQA1*0501	DQB1*0201	DR3	Predisposes
DQA1*0301	DQB1*0302	DR4	Predisposes
DQA1*0301	DQB1*0301†	DQ7	Neutral
DQA1*0301	DQB1*0302	DQ8	Predisposes
DQA1*0102	DQB1*0602†	DQ6	Protects
DQA1*0103	DQB1*0603†	DQ18	Protects

†The DQB1 polymorphisms which carry the codon encoding Asp at position 57 of the β-chain.

* Adapted from Bain SC, 1997

1.2.1. The humoral autoimmune response

Autoantibodies specific for islet antigens have been detected in IDDM patients. At the time that an individual is diagnosed with IDDM or even during the preclinical stage of the disease, serum detection of anti-38kD, anti-bovine serum albumin (BSA), anti-carboxypeptidase, anti-glutamate decarboxylase (GAD), islet cell antibodies (ICA), islet cell surface antibodies (ICSA), and insulin autoantibodies (IAA) is possible. While it has been reported that ICSA are cytotoxic to β-cells in the presence of complement (Dobersen et al., 1980) the disease could not be adoptively transferred by ICA positive sera nor could plasmapheresis of ICA-positive prediabetics alter the progression to IDDM (Harrison, 1992). Furthermore, islets transplanted from an identical twin exhibit a recurrence of insulitis in the absence of ICA (Tarn et al., 1988). The body of evidence rules against a significant role for islet reactive autoantibodies in the pathogenesis of IDDM but this does not diminish their importance as predictive and diagnostic tools.

1.2.1.1. Islet cell antibodies (ICA)

ICA can be detected by immunofluorescence in >60% of recent onset IDDM patients (Kolb et al., 1988a) and first degree relatives who progress to clinical IDDM (Ziegler et al., 1989). High ICA titer is predictive for IDDM but the measure is not absolute as there are ICA positive individuals that do not progress to overt IDDM. The ICA target is likely a glycolipid with a sialic acid moiety (Colman et al., 1988). The possible autoantigen candidates are GT3 (Gillard et al., 1989) and an uncharacterized ganglioside that migrates between GM2 and GM1 upon separation by thin layer chromatography (TLC) (Dotta and Mario, 1996).

1.2.1.2. Insulin autoantibodies (IAA)

As with ICA, IAA can be detected in >60% of recent onset IDDM patients (Palmer et al., 1983a; Wilkin et al., 1985). In ICA positive individuals the additional presence of IAA represents a greater risk of progression to IDDM and hence a greater predictive value for the combinatorial detection (Atkinson et al., 1986). Insulin was the first

and most obvious candidate for a β-cell specific autoantigen. Oral administration of insulin has been reported to slow development of IDDM in the NOD mouse but an etiological role for IAA has not been ascribed (Zhang et al., 1991).

1.2.1.3. Glutamic acid decarboxylase (GAD) autoantibodies

Autoantibodies against a 64kD islet protein now identified as GAD were detected with high specificity in the majority of IDDM sera (Baekkeskov et al., 1990b; Baekkeskov et al., 1987; Baekkeskov et al., 1982). These autoantibodies have been suggested as useful early markers of IDDM. Underlying its importance as an autoantigen, it has been shown that there is a degree of sequence homology between GAD and the coxsackievirus B protein 2C (p2C)(Kaufman et al., 1992; Richter et al., 1994). There is evidence that supports crossreactivity between antibodies raised against purified GAD with antibodies raised against p2C (Hou et al., 1994) as well as crossreactivity to synthetic homologous peptides (Lonnrot et al., 1996). The relevance and veracity of this crossreactivity is, however, still a matter of some dispute (Vreugdenhil et al., 1999).

It is worth noting that autoantibodies against GAD have also been found in non-obese diabetic (NOD) mice, a murine model of spontaneous diabetes (Kaufman et al., 1993; Tisch et al., 1993).

1.2.1.4. 38kDa protein autoantibodies

Autoantibodies against a 38kD islet protein have been detected in the sera of IDDM patients (Baekkeskov et al., 1982). In mice infected with cytomegalovirus (CMV) antibody crossreactivity was observed against a 38kD islet derived protein (Pak et al., 1990). In the sera of uninfected diabetes prone biobreeding (DPBB) rats autoantibodies against a 38kD protein were also observed (Ko et al., 1991).

1.2.1.5. Carboxypeptidase H (CPH) autoantibodies

CPH is a molecule expressed in β-cell secretory granules. It is thought to be involved in the cleavage of proinsulin. CPH autoantibodies have been detected in 40% of prediabetic individuals (Castano et al., 1991).

1.2.1.6. Bovine serum albumin (BSA) autoantibodies

Anti-BSA antibodies have been found in IDDM patients (Martin et al., 1991). A 17 amino acid peptide (ABBOS) extending from position 152 to position 168 was found to bind this antibody which also crossreacts with p69, a β-cell surface protein that may be the target antigen for milk-induced β-cell-specific immunity (Karjalainen et al., 1992).

1.2.2. The cell-mediated autoimmune response

A primary β-cell autoantigen(s) target and the detailed analysis of the initiating events in the autoimmune response in IDDM have yet to be defined. The body of evidence stands heavily in favour of a cell-mediated autoimmune response as the driving force in the pathogenesis of IDDM. Histological examination of pancreas biopsies from recent onset IDDM patients has revealed that the onset of clinical disease is associated with mononuclear cell infiltration of the pancreatic islet (Gepts and Lecompte, 1981a). The insulitis lesion in recent IDDM patients is also characterized by activated T-cells

expressing the major histocompatiblity complex (MHC) class II antigen and interleukin-2 (IL-2) receptors (al-Kassab and Raziuddin, 1990; Alviggi et al., 1984). Further studies have shown that islets from acute diabetics contain macrophages, NK cells, and both CD4 and CD8 positive T-cells (Bottazzo et al., 1985; Hanninen et al., 1992a; Itoh et al., 1993a).

Islet reactive T-cells have been detected in the peripheral blood of recent onset IDDM patients (Shimizu et al., 1993). T-cell responses against GAD65 and porcine proislets have also been detected in both recent onset IDDM patients and prediabetics (Atkinson et al., 1992; Harrison et al., 1991).

The majority of information on islet reactive T-cells is derived from study of the NOD mouse. A testament to the importance of T-cells in IDDM pathogenesis was the report that neonatal thymectomy in NOD mice can prevent diabetes (Ogawa et al., 1985a) while the converse injection of splenocytes from diabetic mice was able to transfer the disease into irradiated mice (Wicker et al., 1986a). Much of our understanding in this area has been made possible by the generation of a number of islet-reactive T-cell clones (Haskins and McDuffie, 1990; Nagata and Yoon, 1992; Nakano

et al., 1991; Shimizu et al., 1993). The diabetogenicity of some of these clones has been demonstrated upon transfer into the irradiated adult (Haskins and McDuffie, 1990) or non-irradiated NOD young (Shimizu et al., 1993). A popular strategy for analyzing the impact of diabetogenic T-cells has been to utilize the TCR sequence from clones such as these to generate transgenic mice (Katz et al., 1993; Schmidt et al., 1997; Verdaguer et al., 1997). The effect of the TCR can then be assessed in relative isolation compared to the heterogeneity and complexity of the normal immune system.

1.2.2.1. Macrophages

Macrophages have been detected at the earliest stages of insulitis (Lee et al., 1988). Pretreatment with silica has been shown to prevent the progression of both insulitis and overt diabetes. A comprehensive investigation of the events initiating the recruitment of macrophages is far from complete. It has been reported, however, that inactivation or depletion of macrophages can alter the course of encephalomyocarditis virus induced diabetes (Baek and Yoon, 1991; Hirasawa et al., 1999; Hirasawa et al.,

1997; Hirasawa et al., 1996). The implication that autoimmune phenomena are not only present but necessary in this model of viral diabetes is a departure from the prevalent paradigm.

It was once suggested that macrophage infiltration could be the consequence of a chemotactic effect of insulin towards activated macrophages (Leiter, 1987). Though the importance of the macrophage in IDDM is undisputed our understanding of its role could do with improvement. The requirement for macrophages in the early insulitis lesion may indicate that one of their relevant functions may be autoantigen presentation to the T-cells that subsequently infiltrate the islet. Another possibility is that activated macrophages may destroy β-cells directly via the production and release of free radicals, i.e. superoxide anion, hydrogen peroxide and nitric oxide (NO) (Horio et al., 1994; Kroncke et al., 1991). A further consideration is the potential for direct destruction via macrophage produced cytokines such as interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α) (Mandrup-Poulsen et al., 1986; Sjoholm, 1998)

1.2.2.2. T-cells

The role of T-cells in the destruction of pancreatic β-cells is potentially twofold. They may either exert direct toxic effects on β-cells and/or modulate the activity of other effector cells. The former function is classically ascribed to CD8+ T-cells while the latter function is usually associated with CD4+ T-cells. Both T-cell subsets are involved in the destruction of pancreatic β-cells (Bach, 1995).

Cloned T-cells bearing both the CD4 and the CD8 co-receptor non-concurrently, have been isolated from the islets of NOD mice. It has been shown by some that CD4+ T-cell lines or clones are capable of inducing diabetes alone (Haskins and McDuffie, 1990; Nakano et al., 1991; Shimizu et al., 1993; Wegmann et al., 1993). Others have demonstrated that both CD4+ and CD8+ are required induce disease (Maugendre et al., 1993; Nagata and Yoon, 1992; Reich et al., 1989). Reports also exist of CD8+ T-cell mediated transfer of diabetes in the absence of CD4+ T-cell co-transfer (Utsugi et al., 1996; Wong et al., 1996). In NOD mice deficient for the expression of β2 microglobulin

(β2m) the development of CD8+ T-cells is consequently impaired. These mice do not develop diabetes, a result that underscores the importance of CD8+ T-cells in the pathogenesis of this disease (Serreze et al., 1994).

1.2.2.2.1. T_H1 vs. T_H2

Helper T-cells (Th) can be separated into at least 2 broad subtypes. The distinction is made primarily on the basis of the cytokine profile of each subtype. Classic Th1 cells produce IL-2 and IFN-γ, and are linked to the initiation and propagation of cell-mediated immune responses. Th2 cells produce IL-4, IL-5, and IL-10, and are associated with humoral immune responses (Mosmann and Coffman, 1989; Seder and Paul, 1994). The cytokine milieu is an important deciding factor in Th cell fate. The presence of IL-12, for instance, favours the development of Th1 cells (Hsieh et al., 1993; Seder et al., 1993) while the presence of IL-4 promotes the development of Th2 cells (Gross et al., 1993; Kopf et al., 1993). Cytokines are also important at the level of Th cell activity. IFN-γ can inhibit the production of Th2 cytokines while IL-4 and IL-

10 have a similar effect on the production of characteristic cytokines by Th1 cells (Abehsira-Amar et al., 1992; Maggi et al., 1992; Tanaka et al., 1993).

Th1 and Th2 cells are both present within the insulitis lesion (Anderson et al., 1993; Shehadeh et al., 1993) but it is the activity of Th1 cells which is of paramount importance in the pathogenesis of IDDM. Transfer of the Th1 subset from a transgenic mouse expressing a diabetogenic TCR into young NOD mice resulted in the transfer of diabetes while transfer of the Th2 subset bearing the identical TCR could only transfer insulitis (Katz et al., 1995). The importance of Th1 cells in IDDM development was underscored by the report that Th1 promoting IL-12 treatment could accelerate diabetes in NOD mice (Trembleau et al., 1995).

1.2.3. Effector Mechanisms

1.2.3.1. Nitric Oxide (NO)

A great deal has been made of the production of NO by macrophages. This free radical is the product of the catalysis of L-arginine to citrulline and NO via the action

of nitric oxide synthase (NOS). Of NOS, there are two types, constitutive (cNOS) and inducible (iNOS). We will concern ourselves with the latter. When brought into play iNOS can trigger the release of large amounts of NO within 8 hours (Marletta, 1993). NO causes the fragmentation of DNA which is followed by eventual cell destruction (Fehsel et al., 1993). Levels of NO are elevated in the islets of NOD mice (Corbett et al., 1993) and when such mice were treated with cyclophosphamide, a chemical known to accelerate diabetes, the expression of iNOS was found to be enhanced (Rothe et al., 1994). Furthermore, the cytotoxic ability of macrophages was inhibited concomitantly with the inactivation of iNOS (Kroncke et al., 1991). The action of NO is not solely linked to the macrophage, however. IL-1 is capable of inducing the release of NO directly from the β-cell of the rat. In this manner NO has been shown to mediate IL-1 facilitated perturbation of normal β-cell function (Corbett et al., 1992; Sjoholm, 1998).

1.2.3.2. Cytokines

The interaction between immune cells is mediated by low molecular weight protein hormones known as cytokines.

The effect of these molecules is often pleiotropic, i.e. a different effect can be elicited dependent primarily on the nature of the target cell. Their production is inducible with little or no constitutive expression and their half-life is relatively short. The principal role of cytokines is to assist in the regulation of immune effector cell development though they are known to possess direct effector function.

The classic Th1 cytokines, i.e. IL-2 and IFNγ, are associated with the destruction of pancreatic β-cells and the development of diabetes while the opposite is true of Th2 cytokines such as IL-4 and IL-10 (Rabinovitch and Suarez-Pinzon, 1998). IFN-γ expression in murine β-cells was capable of inducing diabetes through either direct cytotoxity (Sarvetnick et al., 1988; Sarvetnick et al., 1990) or via promotion of MHC I and adhesion molecule expression, mediating homing of macrophages and T-cells into the islet (Pankewycz et al., 1995). Treatment of NOD mice with α-IFN-γ antibody is capable of preventing diabetes development (Debray-Sachs et al., 1991). Murine β-cell expression of IL-2 promotes diabetes in the absence of a lymphocytic infilitrate (Heath et al., 1992) while TNF-α

has the converse effect, inducing infiltration but not diabetes (Grewal et al., 1996; Higuchi et al., 1992; Picarella et al., 1993).

The administration of IL-4 systemically was shown to prevent diabetes in NOD mice through the preferential development of CD4+ Th2 cells (Cameron et al., 1997). Expression of IL-4 in the β-cells of NOD mice likewise prevented diabetes (Mueller et al., 1996). Though a Th2 cytokine, IL-10 has failed on numerous occasions to alter the progression of diabetes in mice. Transgenic expression of IL-10 in the β-cells of NOD mice did not have an ameliorating effect on diabetes incidence (Lee et al., 1996). The diabetes incidence rate in mice transgenic for IL-10 and lymphocytic choriomenigitis virus (LCMV) glycoprotein (GP) expression in the β-cell was not different from the diabetes incidence in LCMV-GP single transgenic mice (Lee et al., 1994). If, however, IL-10 is fused to a Fc fragment and administered to NOD mice systemically diabetes can be prevented (Zheng et al., 1997).

Evidence exists that IL-1 receptors are present on the surface of β-cells (Eizirik et al., 1991). It is now suspected that the previously reported IL-1 mediated β-cell

toxicity (Palmer et al., 1989; Sandler et al., 1991) occurs via a direct interaction. In furtherance of this contention it has been demonstrated that IL-1 receptor antagonist treatment of previously diabetic NOD mice receiving a syngeneic islet transplant inhibited recurrence of diabetes (Sandberg et al., 1997). NO has been implicated in IL-1 mediated destruction of murine islets (Southern et al., 1990) but more recent work with human islets indicates that an NO independent mechanism may predominate in the destruction of human β-cells (Delaney et al., 1997; Eizirik et al., 1994).

Other cytokines such as IFN-α (Stewart et al., 1993) and IL-12 (Hsieh et al., 1993) have been shown to promote insulitis and diabetes.

1.3. Environmental factors

At this juncture the genetic influence upon the development of IDDM is not a matter of great dispute. The extent to which the genetic makeup of an individual contributes to diabetes risk is best illuminated by a number of twin studies which detail a concordance risk ranging between 30 and 50% (Barnett et al., 1981; Gottlieb

and Root, 1968; Lo et al., 1991; Olmos et al., 1988; Risch, 1987). It has been proposed that the concordance rate between twins is likely less than 40% for a number of reasons: a) twins are more apt to be exposed to the same factors in the environment than unrelated individuals b) identical twins concordant for diabetes are influenced to a greater extent by population calls than are diabetes disconcordant twins c) a large portion of twins which carry the entire known complement of diabetes predisposing genes are together non-diabetic (Bach, 1994; Johnston et al., 1983). The implication is that, of those individuals with a genetic predisposition for diabetes, most will not develop the disease. Factors in the environment are thus, more than likely contributors to the pathogenesis of IDDM. The factors most often reported are viruses and dietary constituents.

1.3.1 Viruses

The list of viruses associated with IDDM in both humans and animals is extensive (Table 4). The evidence linking these factors is primarily epidemiological. Seasonal and geographical variations, indicative of an

Table 4* Viruses implicated in IDDM pathogenesis

Virus	Host	Involvement of genetic factors	Remarks
RNA viruses			
Picornaviridae			
Coxsackie B	Mice	+	Virus was passed in murine B cells before infection Cytolytic destruction of B cells leading to IDDM
	Non-human primates	+	Virus was passaged in monkey B cells before infection Development of transient IDDM
	Humans	ND	Evidence from epidemiological studies, anecdotal reports Virus identified and isolated from pancreas of IDDM patients shown to be diabetogenic in mice
Encephalomyocarditis	Mice	+	Cytolytic destruction of B cells leading to clinical diabetes
	Hamster	+	
Mengovirus	Mice	+	Cytolytic destruction of B cells
Foot-and-mouth disease virus	Pigs, cattle	ND	
Retroviridae			
Retrovirus	Mice	+	B-cell-specific expression of retroviral genes associated with development of insulitis and IDDM in NOD mice
	Humans	ND	Retroviral particles and genome identified in B cells of IDDM patients Antibodies against specific retroviral antigens (including some that crossreact with insulin) in IDDM patients and their close relatives
Togaviridae			
Rubella	Hamsters	ND	Possible association with autoimmune IDDM
	Rabbits	ND	
	Humans	ND	Possible association with autoimmune IDDM, especially congenital rubella syndrome
Bovine viral diarrhoea-mucosal disease virus	Cattle	ND	Suspected autoimmune responses
Paramyxoviridae			
Mumps virus	Humans	+	Possible induction of islet-cell autoantibodies
Reoviridae			
Reovirus	Mice	+	Possible association with autoimmunity and diabetes in mice
DNA viruses			
Parvoviridae			
Kilham's rat virus	Rats	+	No distinct infection of rat B cells Development of B cell-specific autoimmunity, leading to IDDM
Herpesviridae			
Cytomegalovirus	Humans	ND	Association with autoimmune IDDM
Epstein-Barr	Humans	ND	Possible induction of autoimmune IDDM
Varicella zoster	Humans	ND	Indirect evidence of an association with IDDM

ND, not determined

* Adapted from Yoon, 1997

infectious agent, have been reported in the rates of diabetes incidence worldwide (Adams, 1926; Maugh, 1975; Szopa et al., 1993; Yoon, 1990). Clinical case reports detailing the development of diabetes subsequent to documented viral infections are evident in the academic record (Yoon, 1990). Viruses have also been isolated from the pancreata of recently deceased acute diabetics (Yoon, 1993).

1.3.1.1. Causative agents

The means by which viruses might cause the destruction of pancreatic β-cells are twofold. A direct infection of β-cells has the potential consequence of cytolysis. A second possibility is the indirect viral triggering of an immune response that assumes an autoimmune aspect independent of its role in clearance of the virus (Yoon, 1993). This dissertation is focused on retrovirus mediated pathogenesis of IDDM, either through the direct action of endogenous retroviruses or through the action of retroviral reverse transcriptase on either EMC and/or CB4 virus specifically.

1.3.1.1.1. Encephalomyocarditis (EMC) virus

The best evidence for a viral etiology in IDDM comes from studies of EMC virus infection in mice. EMC belongs to the family picornaviridae, which is characterized by its small size (~20nm diameter), non-encapsulated icosahedral structure, and single-stranded, linear RNA genome. It was shown that the M variant of EMC is capable of inducing a diabetes-like syndrome in genetically susceptible strains of mice but in an inconsistent manner (Craighead and McLane, 1968; Ross et al., 1976; Yoon et al., 1977). Two further variants were isolated, one which induced diabetes in >90% of infected mice (EMC-D), and the other which was completely non-diabetogenic (EMC-B) (Yoon et al., 1980; Yoon et al., 1982). A comparison of the complete nucleotide sequences of both variants revealed a 14 nucleotide difference (Bae et al., 1989a; Bae et al., 1989b). Examination of a series of viral mutants generated from EMC-B and EMC-D showed that a guanine (G) at position 3155, encoding for alanine-776 in viral capsid protein 1 (VP1), is found in all diabetogenic variants. An adenine (A) in the same position, resulting in a threonine residue, is found in all non-diabetogenic variants. The 776^{th} amino acid

lies in a conserved hydrophilic patch in VP1, a region which has been shown to be critical in the attachment of EMC to pancreatic β-cells (Bae and Yoon, 1993; Baek and Yoon, 1991; Eun et al., 1988).

Susceptibility to EMC-D is dependent on genetic factors in the host animal (Yoon et al., 1976; Yoon and Notkins, 1976). Of the inbred strains so far tested only SJL/J, SWR/J, DBA/1J and DBA/2J strains were susceptible while C57BL/6J, CBA/J and AKR/J were resistant (Yoon et al., 1980). F1(SJL/J x C57BL/6J) were resistant to EMD-D as were F2(F1 x C57BL/6J). However, 48% of EMC-D infected F2(F1 x SJL/J) and 24% of F2(F1 x F1) became diabetic (Baek and Yoon, 1991). This pattern of segregation in the susceptibility phenotype indicates that the inbred strains carry an autosomal recessive gene that is inherited in a Mendelian fashion.

Initial studies suggested a role for T-cells in the destruction of murine pancreatic β-cells by EMC-D (Buschard et al., 1983). Depletion of lymphocytes, however, did not have an effect on the progression of diabetes in this model (Yoon et al., 1985). Virus infected nude mice exhibit a response identical to the diabetes occurring heterozygous littermates. The treatment of virus infected mice with

cyclosporin A had the opposite of the expected effect; the incidence and severity of diabetes was enhanced (Vialettes et al., 1983).

1.3.1.1.2. Coxsackie B4 (CB4) virus

There is a large body of anecdotal as well as formal epidemiological evidence linking human IDDM with Coxsackie B virus infections (Yoon and Kominek, 1996). Statistically significant differences have been repeatedly detected between acute diabetics and non-diabetics. Unfortunately, as many studies exist which assert either no difference or even paradoxically elevated levels of anti-CB4 antibodies in normal controls compared to acute diabetics. A factor that likely obscures the relationship between the presence of diabetes and that of anti-CB4 antibodies is the number of CB4 variants. The B4 designation is a serotype and at least 13 variants have been isolated (Prabhakar et al., 1982). The results of another study showed that of four B4 variants tested only one was capable of inducing diabetes in mice (Yoon et al., 1986). Therefore, if a CB4 linked etiology for human IDDM is to be believed then the following scenario must be considered: If a population is

exposed to and develops antibodies to a non-diabetogenic variant of CB4, epidemiological studies which draw subjects from this population will not discover a link between diabetes and anti-CB4 antibody positivity. In order for a diabetogenic CB4 variant to be positively linked to diabetes in such studies it is a likely necessity that the virus infect a virgin population which does not possess immunity derived from previous infection with non-diabetogenic CB4 variants. Such immunity has previously been demonstrated in a related model of viral diabetes in which infection of the EMC-B strain prior to infection with the diabetogenic EMC-D strain can confer protection from diabetes (Notkins and Yoon, 1982). In this model we are subscribing to the probable assumption that the diabetogenic variant(s) are rare (Yoon et al., 1986).

Coxsackie B viruses have been isolated from the pancreata of recent onset IDDM patients (Champsaur et al., 1982; Gladisch et al., 1976; Yoon et al., 1979). These viruses were found to induce murine diabetes in a mouse strain specific manner. In one instance SJL/J males were susceptible to Coxsackie induced diabetes while CBA/J, C57BL/6J, and Balb/c were resistant (Yoon et al., 1979). In another case the virus isolate was capable of inducing

glucose intolerance in SJL/J and DBA/2 mice but not in Balb/c nor C3H mice (Champsaur et al., 1982).

Naturally occurring Coxsackie B virus pancreatic infections usually produce acinar cell pancreatitis and initial studies have demonstrated that CB4 did not induce diabetes upon inoculation into mice (Ross et al., 1974). The diabetogenic ability of the virus was enhanced through serial passage through heterogeneous pancreatic β-cell cultures (Yoon et al., 1978). Regardless, a pure β-cell tropic virus was difficult to isolate because the passaging cultures were made up of mixed cell types (Toniolo et al., 1982; Yoon et al., 1978). As with EMC-D the ability of CB4 to induce diabetes is dependent upon the host animal's genetic background. SJL/J, SWR/J, and NIH Swiss mice have been shown to be diabetes susceptible upon infection while other inbred strains such as C57Bl/6J, CBA/J, AKR, Balb/c, C3H/J, DBA/1J and DBA/2J were diabetes resistant. The sex of the infected mouse is also a determining factor in both the severity and incidence of diabetes; males are more likely to become hyperglycemic and to a greater degree (Yoon et al., 1978). The remaining 5 B serotypes of Coxsackie virus have also been analyzed for diabetogenicity. It was shown, in a similar manner, that

serial passage of each of these strains could produce β-cell tropism and a diabetogenic effect (Toniolo et al., 1982).

Genetic studies have demonstrated that presence of the diabetic "db" gene mutation on chromosome 4 results in susceptibility to CB4 infection (Loria et al., 1984; Webb et al., 1976). The nature of the primary defect was reported to be an impaired humoral response to CB4 infection, specifically an inadequate level of IgM and IgG anti-CB4 antibodies (Loria et al., 1986; Montgomery and Loria, 1986). A deficiency in lymphocyte numbers in the spleen has also been found (Montgomery et al., 1990).

Expression of GAD has been estimated to increase by two to three-fold prior to the onset of hyperglycemia in SJL/J and CD1 mice infected with CB4 (Baekkeskov et al., 1990a; Gerling et al., 1988). It was also demonstrated that 90% of mice infected with CB4 are positive for anti-CB4 antibodies within 6 weeks (Gerling et al., 1991). The use of polyclonal antisera against GAD has shown that the level of GAD65 is three times greater in CB4 infected mice compared to uninfected mice (Hou et al., 1993). A similar experiment conducted in genetically resistant mice revealed that levels of pancreatic GAD in these animals was not elevated (Gerling and Chatterjee, 1993).

Plaque purification of the Edwards isolate, a CB4 virus of human origin, resulted in the generation of 3 strains designated E1, E2, and E3 (Hartig et al., 1983; Hartig and Webb, 1983). The diabetogenic E2 strain, which demonstrates a more pronounced accumulation in murine islets, has been the focus of a number of subsequent experiments.

A comparison of the nucleotide sequence of the diabetogenic E2 strain (Kang et al., 1994) with the prototypical JVB strain (Jenkins et al., 1987) has illuminated a 111 amino acid difference between the two. Comparative analysis of another diabetogenic strain adapted from JVB has revealed a 7 seven amino acid difference (Titchener et al., 1994). Further comparison of the diabetogenicity of the mutant phenotype is required before the critical regions conferring the ability to induce disease are found.

1.3.1.1.3. Retroviruses

Retroviruses are RNA viruses that replicate via a DNA intermediate. This is a feature of their replication cycle that allows them to integrate into host genomic DNA. These

latent proviruses can be induced, by a triggering event (uv, chemical, secondary infection, etc.), to re-enter the active replication cycle (Varmus and Swanstrom, 1985). A distinction must be made between infectious and endogenous retroviruses. The former are usually limited in their distribution throughout the body while the latter are transmitted vertically, via the germ line, and are present in every body cell. At present there are three retrovirus subfamilies: 1. Oncovirinae 2. Lentivirinae 3. Spumavirinae. Within the first subfamily we will find viruses grouped according to virion structure; A, B, C, and D types of which A and C types are of particular interest to us.

Type A particles or intracisternal type A particles (IAP) are present in high numbers, often within intracellular membranes. They are hollow, spherical structures, which are 60 to 90 nm in diameter and have a double-walled appearance (Coffin, 1990).

Complete intracellular Type C particles are not typically seen. Often, they are first visible at the cell membrane during the budding stage. Newly budded virions appear to have hollow nucleocapsids which become electron dense with condensation of the core as maturation of the particle progresses (Coffin, 1990).

Retroviruses have been implicated in the disease process of a number of autoimmune disorders, ex. systemic lupus erythematosus (SLE) (Phillips, 1979; Pincus, 1982), rheumatoid arthritis (Rodahl and Iversen, 1985), and Graves' disease (Ciampolillo et al., 1989). In addition, autoimmune phenomena have been reported in human immunodeficiency virus-1 patients (HIV) (Krieg and Steinberg, 1990).

Currently, there are three mechanisms by which retroviruses are thought to alter immune function. First, mature particles or retroviral proteins can have direct effects on the immune system. Two infectious retroviruses, radiation leukemia virus and Moloney murine leukemia virus (MMLV), enhance mouse Class I expression on thymocytes (Meruelo et al., 1978; Wilson et al., 1987). The immune consequences in these two cases are, however, uncertain. Second, indirect effects can occur via host immune responses to retroviral proteins. Retroviral expression may have variable effects depending on the genetic background of the host. Simian immunodeficiency virus (SIV), for example, does not cause any morbidity in African monkeys yet infected Asian macaques develop a fatal immunodeficiency (Letvin et al., 1985). In general, the host response to a viral infection leads to the release of interferons which can have non-specific effects that may

contribute to autoimmunity. IFN-γ, for instance, increases MHC Class II expression in tissues normally expressing low amounts of MHC II. This may, in turn, trigger an otherwise inactive T-cell response (Feldmann et al., 1987; Schattner, 1988). Specific host responses leading to autoimmunity are described in models advocating anti-idiotypic antibodies and molecular mimicry. Antibodies to viral cell-surface-binding epitopes may serve as antigens for 'anti-idiotypic antibodies' which can then serve as autoantibodies against the cells which the virus would normally target (Plotz, 1983). With regards to molecular mimicry; sequence homology exists between retroviral proteins and host proteins (Query and Keene, 1987) which raises the possibility of cross reaction following expression of retroviral components. It should be appreciated that an autoimmune response can arise if the humoral response is maintained against the homologous host protein(s). Finally, retroviral proteins can be released from expressing cells and picked up by other 'bystander' cells, targeting them for immune destruction (Siliciano et al., 1988). The pathologic effects would be similar to autoimmune destruction.

The third mechanism by which retroviruses can alter immune function is integration into the host genome. We must distinguish between integration which occurs within the lifespan of an individual and that which is inherited

in the form of endogenous retroviruses. In both instances the site of integration is of great importance. We should note that integration within a cellular gene will likely inactivate that gene, though it may not have much effect phenotypically unless the chromosomal copy of the gene is likewise affected. The end result of retroviral integration is that regulation, expression, and integrity of host and viral gene products may be altered, imbueing the host cell with immunogenic properties (Krieg and Steinberg, 1990).

Type A and C retroviral particles have been identified in NOD islets (Fujino-Kurihara et al., 1985; Fujita et al., 1984; Suenaga and Yoon, 1988) and in the β-cells of genetically diabetic (db/db) mice (Leiter, 1985; Leiter and Bedigian, 1979). Type C particles have been identified in the pancreatic β-cells of two diabetic patients who died shortly after diagnosis. This has given impetus to the study of retroviruses as an etiologic agent in the pathogenesis of Type I Diabetes. The tendency of IDDM to run in families has been extensively studied and the possibility that endogenous retroviruses may play a role in the observed susceptibility to this disease is intrigueing. It has been demonstrated that there is increased expression of p73, a gag-gene product of IAP, in db/db mice (Leiter and Kuff, 1984). This increased expression has been shown to be glucose dependent (Leiter et al., 1986).

The expression of a gag-gene product of Type C retroviruses, p30, has been found to increase with the administration of cyclophosphamide (Nakagawa et al., 1992). Two xenotropic endogenous Type C retrovirus loci have been found in NOD islets but not in diabetes resistant NON (Non-Obese Non-diabetic) islets. It is reported that this xenotropic Type C expression distinguishes NOD from NON ß-cells (Gaskins et al., 1992).

Cross reactivity has been demonstrated between p73 and Insulin in humans, NOD mice, and db/db mice (Hao et al., 1993; Serreze et al., 1988). Evidence from work in our laboratory indicates that vaccination with gp70, a major coat protein of Type C retroviruses, and p73 may prevent the development of diabetes in NOD mice (Pak et al., 1993). It has also been revealed that the administration of cyclophosphamide increases the rate of progression to overt diabetes with severe insulitis in NOD mice (Harada and Makino, 1984; Suenaga and Yoon, 1988). A finding incidental to the rapid occurrence of insulitis and diabetes was the marked presence of retroviral Type A particles. Cyclophosphamide treatment was directly linked with the increased frequency of retroviral particles in β-cells (Harada and Makino, 1984). The expression of retroviral mRNA and the group specific retroviral antigen p73 of IAP has been examined. Comparison of the expression of gag,

pol, and env mRNAs in NOD and ICR mice using in situ hybridization showed that there was a marked difference in the β-cell retroviral expression in these two strains. While roughly 25% of NOD islets were positive for retroviral mRNA no expression was detected in any of the ICR islets analyzed. Similarly anti-p73 antibody, used to examine the expression of the group specific antigen showed that p73 expression was restricted to NOD islets (Pak et al., 1993).

1.3.1.1.4. Other viruses

Individuals afflicted with congenital rubella syndrome (CRS) have a higher incidence of IDDM than the remainder of the population. A number of prospective studies demonstrated that 10-20% of individuals with CRS developed IDDM within 5-20 years (Yoon, 1995). ICA and IAA have been found in 50 to 80% of CRS patients that also had IDDM (Ginsberg-Fellner et al., 1984). Such individuals carry an increased frequency of HLA-DR3 and a conversely decreased frequency of HLA-DR2 (Parkkonen et al., 1992). A case report exists of an individual developing both Still's disease, an acute onset form of systemic rheumatoid

arthritis, and IDDM after an infection with Rubella virus (Sibley, 1990).

Neonatal golden Syrian hamsters infected with β-cell passaged rubella virus develop diabetes within 10 days (Rayfield et al., 1986). The insulitis lesion in these animals is characterized by mononuclear cell infiltration of the islets as well as rubella virus antigen detected by immunofluorescence. The mechanism by which rubella virus induces diabetes is unknown. Insulitis has been found in 34.5% of infected hamster islets and a weak ICA positivity has also been detected. These are findings that support an autoimmune pathogenesis.

Rubella virus belongs to the family Togaviridae. Members of this family are enveloped, with the lipoprotein coat being derived from the host cell membrane as the virus buds through. It is plausible that immune recognition of inserted viral coat proteins or altered/exposed host proteins might be the root of an autoimmune attack against the infected β-cell. Another possibility is autoimmune destruction based on molecular mimicry. Crossreactivity has been demonstrated where a monoclonal antibody which recognizes capsid and envelope proteins also reacts with a

52kD protein in extracts of rat and human islets (Karounos et al., 1993).

The earliest RNA virus to be associated with IDDM was the mumps virus. Two anecdotal reports dating from the 19th century describe the development of IDDM soon after an infection with the mumps virus (Harris, 1899; Rossini et al., 1985). Several cases have been reported in the modern era in which the onset of IDDM has been preceded by an antecedent mumps infection (Gamble, 1980). Experimental infection of a human insulinoma cell line has been reported to trigger the release of IL-1 and IL-6 with concomitant upregulation of MHC class I and II molecules (Cavallo et al., 1992). The upregulation of MHC class I in this model has been independently confirmed (Parkkonen et al., 1992). These reports do not offer definitive evidence implicating mumps virus in the pathogenesis of human IDDM. They are, however, consistent with and suggestive of a role for mumps virus in the propagation of an autoimmune response.

Kilham's rat virus (KRV) has been shown to induce diabetes in an experimental model for IDDM. Diabetes resistant bio-breeding (DRBB) rats do not normally become diabetic but subsequent to administration of KRV up to 30% of infected animals develop clear signs of IDDM (Guberski

et al., 1991). The mechanism by which KRV induces diabetes in this model is an area of active study. There is no evidence of direct infection of pancreatic β-cells by this virus (Brown et al., 1993).

1.3.1.2. Preventive agents

The influence of viruses on diabetes pathogenesis is not restricted to induction of the disease. Reports also exist suggesting that infection by certain viruses such as lymphocytic choriomeningitis virus (LCMV) in NOD mice and DPBB rats (Dyrberg et al., 1988; Oldstone, 1988) and mouse hepatitis virus (MHV) in NOD mice (Wilberz et al., 1991) can prevent development of IDDM.

2. Research objectives

2.1. General objective

The overall objective of this dissertation was to examine the role of viruses in the development of IDDM. The presence of unique endogenous retroviruses and the effects of retrovirus encoded reverse transcriptase on other RNA viruses, i.e. EMC-D and CB4, were examined as possible contributors to IDDM pathogenesis in mice and in humans.

It is clear that IDDM is an autoimmune disease in which the cell-mediated arm of adaptive immunity plays a key role. The genetic makeup of an individual has likewise been recognized as a critical factor. The most contentious sector of diabetes research has, however, centered on the role of environmental factors in IDDM etiology. The epidemiological record reflects the influence of environmental factors as evidenced by diabetes incidence rates mirroring a classic outbreak pattern, i.e. constrained by both time and geography.

Endogenous retroviruses are present in all vertebrate cells as integrated proviruses. Their expression can be induced by environmental stresses, including infection by other viruses. The presence of retroviral structures in the β-cells of diabetes prone mice and antibodies specific for

retroviral antigens in human IDDM patients led researchers to suspect a role for retroviruses in IDDM pathogenesis.

The induction of diabetes in mice by EMC-D virus is the prototypical experimental model for viral IDDM. Early work in the area revealed that direct cytolytic destruction of pancreatic β-cells was possible if animals were infected with a relatively large dose of the virus. More recent evidence suggests that minute amounts of the virus are also capable of inducing diabetes in certain mouse strains in a macrophage dependent manner. The latter situation more closely mimics a naturally occurring infection but less work has been done on the latter model and less is known about the steps between infection and β-cell destruction.

The virus most often associated with human IDDM is CB4. Vast epidemiological data and anecdotal reports link CB4 and IDDM but it remains to be shown whether this virus is responsible for a significant portion of diabetes cases. As with EMC-D, cytolytic destruction of pancreatic β-cells by CB4 has been demonstrated but such cases are thought to be rare.

Convincing evidence has yet to be presented, directly linking retroviruses with β-cell destruction or the

autoimmune cell-mediated responses responsible for β-cell destruction. In this study unique human and murine retroviral sequences were examined for relevance to IDDM pathogenesis. The role of retroviral reverse transcriptase as a factor contributing to the persistence of the diabetogenic RNA viruses, EMC-D and CB4, was also examined.

2.2. Specific objectives

2.2.1. Discovery, characterization and determination of the role of a unique endogenous retrovirus in NOD mice.

2.2.1.1. Generation of a λgt11 cDNA library from an NOD mouse derived β-cell line and selection of λgt11 clones reactive against prediabetic NOD sera.

2.2.1.2. Nucleotide sequence determination and sequence homology analysis of prediabetic NOD sera reactive λgt11 clones.

2.2.1.3. Expression of an endogenous ecotropic murine leukemia viral envelope gene (env) pET30b clone in

Escherichia coli (E.coli) using a histidine 6 fusion system.

2.2.1.4. Generation of recombinant env deletion mutants.

2.2.1.5. Western blotting analysis for env regions critical to specific prediabetic NOD serum antibody reactivity.

2.2.1.6. T-cell proliferation assay for cell-mediated immunity against purified recombinant env protein in NOD mice.

2.2.1.7. T-cell proliferation assay for cell-mediated immunity against env protein expressed by transfection into Balb/c APC's.

2.2.1.8. Comparative analysis of humoral env positivity in NOD mice vs. non-diabetogenic mouse strains

2.2.1.9. Analysis of age dependent transcription of env in the pancreata of NOD mice

2.2.2. Analysis of the candidate IDDM-associated human retrovirus sequence, $IDDMK_{1,2}22$ and evaluation of its potential role in IDDM pathogenesis.

2.2.2.1. Detection of the $IDDMK_{1,2}22$ gene in human plasma by polymerase chain reaction (PCR) analysis.

2.2.2.2. Screening for genomic DNA in critical viral RNA isolates from human plasma.

2.2.2.3. Sequence analysis of $IDDMK_{1,2}22$ specific PCR product.

2.2.3. Determination of the role of endogenous retrovirus mediated reverse transcription on persistence of EMC-D virus as it pertains to the development of IDDM in mice.

2.2.3.1. Characterization of diabetes in EMC-D infected DBA/2 mice.

2.2.3.2. PCR detection of endogenous EMC-D cDNA in the pancreata of EMC-D virus infected DBA/2 mice.

2.2.3.3. Artefactual generation of a specific PCR signal from EMC-D virus or viral RNA under standard and permissive assay conditions.

2.2.3.4. Examination of the effectiveness of RNase A treatment in the prevention of artefactual generation of EMC-D cDNA.

2.2.3.5. Sequence confirmation of PCR product amplified with EMC-D specific primers.

2.2.3.6. Determination of the effect of the reverse transcriptase inhibitor azidothymidine (AZT) on the generation of endogenously reverse transcribed EMC-D RNA viral genome and progression of diabetes in EMC-D infected DBA/2 mice.

2.2.4. Determination of the role of endogenous retrovirus mediated reverse transcription on the persistence of CB4 virus as it pertains to the development of IDDM in humans.

2.2.4.1. Case Report: An IDDM related child mortality.

2.2.4.2. Histological characterization of human pancreatic islets from a recently deceased type I diabetic.

2.2.4.3. Detection of cDNA generated from CB4 viral RNA in the pancreatic tissue of a recently deceased type I diabetic.

3. Materials and methods

3.1. A unique endogenous retrovirus in NOD mice

3.1.1. Mice

Non-obese diabetic (NOD) mice were bred from founders donated by Dr. B. Singh at the University of Alberta in 1989. The new colony was designated NOD/Yn. The incidence of diabetes in NOD mice is influenced by a number of factors including variation in the breeding protocols and endemic environmental factors. It is accepted that various NOD colonies worldwide exhibit differences in the incidence of insulitis and diabetes (Baxter et al., 1989; Pozzilli et al., 1993). At 32 weeks of age the cumulative incidence of diabetes in the NOD/Yn colony is ~75% in females and ~20% in males.

Non-obese non-diabetic (NON), SJL/J, ICR, Balb/c and C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, Maine). All animals were maintained on site at the Animal Resource Centre (ARC) of the University of Calgary under specific pathogen free (SPF) conditions. They were fed autoclaved food and water ad libitum.

3.1.2. The mouse insulinoma 6N8a (MIN6N8a) cell line

MIN6N8a cells were kindly provided by Dr. J. Myazaki (University of Tokyo, Japan). Cells were cultured in a high glucose formulation of Dulbecco's modified eagle medium (HGDMEM) supplemented by 10% fetal bovine, MD), 10ug/ml gentamicin 10mM sodium pyruvate, 20mM L-glutamine (Gibco BRL, Gaithersberg, MD) and 50mM β-mercaptoethanol (Sigma, St. Louis, MO). Cultures were maintained at 37°C under 5% CO_2. Culture medium was changed every 2-3 days.

3.1.3. Construction of λgt11 cDNA library from MIN6N8a cells

mRNA was isolated from cultured MIN6N8a cells using a PolyATract mRNA isolation kit (Promega, Madison, WI). Briefly, cultured MIN6N8a cells were disrupted and resuspended in guanidium extraction buffer. mRNAs with a poly(A) tail were directly bound to biotinylated oligo(dT) primer. After subsequent binding to streptavidin conjugated magnetic beads, mRNAs were detached by low salt buffer.

First strand cDNA was synthesized by avian myeloblastoma virus (AMV) reverse transcriptase (Promega) using a proprietary NotI-oligo(dT) primer: 5'- AAT TCG CGG CCG C T(15) -3'. Second strand cDNA was generated with RNase H and DNA polymerase I. The ends of the double stranded cDNA were trimmed with T4 DNA polymerase and an EcoRI adapter (5'- AAT TCC GTT GCT GTC G -3', 5'- CGA CAG CAA CGG -3') was attached to each end of the trimmed cDNA. The double stranded cDNA was cut with NotI and ligated into the corresponding site in the phage λgt11 Sfi-NotI vector (Promega).

In vitro packaging of phage DNAs and infection into E.coli Y1090 was carried out according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden). Briefly, 0.1ml (10,000 pfu) of recombinant phage was mixed with 0.2ml of an overnight culture of E.coli Y1090. This solution was mixed with 2.5ml of warm top agar solution and poured onto a Luria-Bertani (LB) agar plate. The plate was incubated at 42°C for 3-4 hours and then overlaid with a nitrocellulose membrane saturated in 10mM isopropyl-1-thio-β-D-galactoside (IPTG). Subsequent incubation at 37°C for 3-

4 hours facilitated expression of MIN6N8a proteins and transfer of those proteins to the nitrocellulose membrane.

3.1.4. Immunoscreening of MIN6N8a λgt11 cDNA library

Positive clones were identified by a conventional immunoscreening method. Briefly, nitrocellulose membranes were blocked with 5% skim milk powder for 1 hour after which a 100 fold dilution of pooled sera collected from 12 prediabetic NOD female mice aged 8 weeks was added. Alkaline phosphatase-conjugated α-mouse IgG (Sigma) was then added and the reaction was developed with 5-bromo-4-chloro-3-indoyl-1-phosphate (BCIP) and nitroblue tetrazolium (NBT) (Promega). A second and third screen of picked plaques was carried out according to the same protocol to ensure isolation of a pure clone.

3.1.5. Sequence and homology analysis of positive λgt11 clones

The nucleotide sequence at the 5′ end of each positive clone was determined. DNA from recombinant λgt11 clones was

isolated and purified using a standard procedure from Applied Biosystems (Foster City, CA). DNA from an overnight culture of infected E.coli Y1090 was isolated by phenol/chloroform extraction and precipitated with ethanol. After resuspension in distilled water a second precipitation was carried out using 4M NaCl and polyethlyene glycol 8000 (PEG_{8000}). DNA was resuspended in 20uL of distilled water. DNA templates were labeled for cycle sequencing using the Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). Briefly, a reaction was prepared with 9.5ul 4x reaction premix, 5ul double stranded DNA template, and 3.2 pmol of sequencing primer (λgt11 forward primer: 5'- GGT GGC GAC GAC TCC TGG AGC CCG - 3') in a final volume of 20ul. The resulting reaction mixture was overlaid with a drop of mineral oil and PCR amplified under the following conditions: 25 cycles of 96°C for 30sec, 50°C for 15sec, 60°C for 4min. The 4x reaction premix was made up with 16ul 5x terminator ammonium cycle sequencing (TACS) buffer (400mM Tris-HCl, 10mM $MgCl_2$, 100mM $(NH_4)_2SO_4$ pH 9.0), 4ul dNTP mix (750uM dITP, 150uM dATP, 150uM dTTP, 150uM dCTP), 4ul DyeDeoxy A terminator, 4ul DyeDeoxy T terminator, 4ul DyeDeoxy G terminator, 4ul

DyeDeoxy C terminator and 2ul AmpliTaq DNA polymerase (8U/ul). The completed sequencing reactions were then purified using Centri-Sep spin purification columns (Princeton Separations, Adelphia, NJ). 20ul of the completed sequencing reaction was applied and the column was spun at 1300xg for 2 min. The purified sequencing reaction was ethanol precipitated and lyophilized in a Savant Speedvac. The prepared reaction was analyzed by the University of Calgary DNA sequencing laboratory using an Applied Biosystems 373A DNA sequencer.

The identity of cloned sequences was determined by BLAST search of the GenBank database.

3.1.6. Expression of clone 3-2 retroviral envelope protein in E.coli

For expression of the newly identified retroviral envelope protein, λgt11 clone 3-2 was cut with EcoRI and NotI. The cDNA insert was subcloned into vector pET30b (Novagen, Madison, WI) which was subsequently transformed into E.coli BL21 (DE3). Protein expression was induced by addition of 0.5mM IPTG. The expressed proteins were then purified with a Ni-NTA affinity purification system

(Qiagen, Hilden, Germany). Briefly, transformed E.coli were sonicated following IPTG induction. The insoluble fraction was separated by differential centrifugation (10,000xg, 10 min) and resuspended in 6M guanidine HCl denaturing buffer. The soluble fraction was diluted and loaded directly onto Ni-NTA agarose under denaturing conditions according to the manufacturer's instructions. Ni-NTA bound protein was detached by employing a pH gradient. The purified 3-2 retroviral envelope protein was used for T-cell proliferation assay (Figure 6).

3.1.7. Generation of clone 3-2 deletion mutants

Deletion mutants of pET30b clone 3-2 were generated in order to determine the epitopes critical to humoral reactivity. The 3' end of the 3-2 sequence was cut with PstI and sequentially deleted with endonuclease Bal31. Generation of deletion mutants truncated at the 5' end was accomplished by subsequent PCR amplification of the newly generated deletion mutant linear DNA from clone 3-2-47 with different 5' primers (for clone 3-2-65: 5'- GAG AAT TCT GCC ATG CAC GAT GAC CTT AA -3'. clone 3-2-85: 5'- GAG AAT TCA GAA GTT GAA AAG TCC ATC ACT AA -3'. clone 3-2-109: GAG AAT

TCT CTA GAA AAA TCT TTG ACC TCC T -3'.). Truncated pET30b clone 3-2 constructs were self ligated and the resulting deletion mutants were transformed into E.coli and expressed as in section 3.1.6..

3.1.8. SDS-PAGE separation and western blotting of expressed proteins

Expressed proteins from pET30b clone 3-2 and deletion mutants were visualized by SDS-PAGE. Proteins were solubilized in SDS-PAGE loading buffer (75mM Tris pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% sucrose, 0.002% bromophenol blue). The suspended proteins were separated on 10 or 15% PAGE. The gel was visualized by staining with a 0.1% solution of coomassie blue dye.

For western blotting the gel run proteins were transferred to nitrocellulose membrane via electrotransfer. The membrane was blocked with 5% skim milk and a 100 fold dilution of pooled sera collected from 12 prediabetic NOD female mice aged 8 weeks was added. Alkaline phosphatase-conjugated α-mouse IgG (Sigma) was then added and the reaction was developed with 5-bromo-4-chloro-3-indoyl-1-phosphate (BCIP) and nitroblue tetrazolium (NBT) (Promega).

3.1.9. T-cell proliferation assay for reactivity to administered retroviral envelope protein

Cell-mediated immunity against recombinant 3-2 retroviral envelope protein was examined by T-cell proliferation assay. Spleens were harvested from 10 week old prediabetic NOD mice (n=6) and splenocytes were prepared by grinding the tissue in hemolysis buffer (0.15M NH_4Cl, 0.01M $KHCO_3$, 0.01M EDTA) followed by centrifugation (400xg, 5min). T-cells were purified using a commercial column (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. APC's were prepared from the whole splenocyte fraction by γ-irradiation (2000 Rad). A T-cell/APC mixture at a ratio of 1:4 was cultured in RPMI 1640 with 10ug/ml recombinant 3-2 retroviral envelope protein under 5% CO_2 at 37°C for 3 days. The culture was harvested by an automated cell harvester after a 16hr pulse with 1uCi of ^3H-thymidine (Amersham, Arlington Heights, IL). The stimulation index was determined by calculating the ratio between the sample counts per minute (cpm) value and the cpm of the HSA negative control from the same assay.

3.1.10. T-cell proliferation assay for reactivity to endogenously expressed MuLV envelope protein.

The complete MuLV envelope gene was subcloned into the EcoRI/NotI site of the eukaryotic expression vector pcDNA3. The resulting construct pEnv was transfected into the T-cell depleted fraction of Balb/c splenocytes that were prepared as in section 3.1.9.. T-cells were depleted by incubation of the whole Balb/c splenocyte fraction with 10ng/ml rabbit-α-mouse α-CD3 antibody(Sigma) and subsequent treatment with a 10 fold dilution of rabbit complement (Sigma). The resulting APC's were transfected with pEnv by electroporation (1000uF, 250v) and incubated at 37°C for 2 days to facilitate transient expression of the envelope gene. APC's were γ-irradiated (2000 Rad) before use in the T-cell proliferation assay. T-cells were isolated as in section 3.1.9.. A T-cell/APC mixture at a 1:1 or 3:1 ratio was cultured under 5% CO_2 at 37°C for 2 days. Proliferation was assayed as in section 3.1.9..

3.1.11. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of retroviral envelope gene expression

Gene expression of the 3-2 retroviral envelope gene was examined in NOD female mice (n=5-10) at different ages. Pancreata were harvested from neonatal as well as 4, 8, 12, 16, and 20 week old mice. Total RNA was isolated using a modified acid guanidium phenol chloroform (AGPC) method (Monstein et al., 1995). Briefly 25mg of pancreatic tissue was homogenized in guanidium thiocyanate (GTC) solution (4M guanidium thiocyanate, 0.5% N-lauroylsarcosine, 10mM Tris-HCl pH 7.5, 300mM β-mercaptoethanol) and extracted with phenol-chloroform. The precipitated pellet was resuspended in guanidine solution containing 6M guanidine·HCl, 25mM EDTA pH 7.0, 10mM β-mercaptoethanol, 0.5% SDS, 0.5mg/ml proteinase K. This solution was extracted once again with phenol-chloroform. The precipitated and dried RNA pellet was treated with RNase-free DNase I (Boehringer Mannheim, Laval, QC) at a final concentration of 1U/ul and extracted a final time with phenol-chloroform. The dried total RNA pellet was resuspended in 50ul of diethylpyrocarbonate (DEPC) treated water. First strand cDNA was generated from 1ug of total RNA using oligo(dT) primer as in section

3.1.3.. PCR was carried out with primers specific for the MuLV envelope gene (forward: 5'- GAA GTT GAA AAG TCC ATC ACT AAT CTA G -3'. backward: 5'- CCA GGG CCT GCA CTA CCG AAA TCC TG -3') and for mouse β-actin (forward: 5'- CAT GTT TGA GAC CTT CAA CAC CCC -3'. backward: 5'- GCC ATC TCC TGC TCG AAG TCT AG -3') under the following conditions: for MuLV envelope gene 35 cycles of 95°C for 30sec, 60°C for 30sec, 72°C for 60sec, and for mouse β-actin 25 cycles under the same conditions. The amplified PCR products were visualized on 1% agarose by ethidium bromide staining.

3.2. The role of the retroviral sequence IDDMK$_{1,2}$22 in the pathogenesis of human IDDM

3.2.1. Subjects

We collected 3 to 7 ml of blood in vacutainer tubes, using K_3EDTA as an anti-coagulant, from recent onset IDDM patients (n = 13) and normal control subjects (n = 10).

3.2.2. Isolation of genomic DNA, cytoplasmic RNA, and viral RNA.

We reserved 200ul of whole blood for isolation of genomic DNA using the QIAmp Blood Kit (Qiagen, Ontario, Canada) and the remainder was used for the isolation of lymphocytes using Ficoll-Paque centrifugation (Pharmacia Biotech, Uppsala, Sweden). Cytoplasmic RNA was isolated from the lymphocytes after separation from the nuclei, according to a RNeasy protocol (Qiagen). Viral RNA was isolated from 140ul of plasma using the QIAmp Viral RNA kit (Qiagen).

3.2.3. Reverse transcription of cytoplasmic and viral RNA

Cytoplasmic RNA (10.5 ul) from the 50 ul of total RNA isolated from the lymphocytes and viral RNA (8 ul) from the 50 ul of isolate obtained from the plasma was reverse transcribed using a poly(A) or oligo$(dT)_{12-18}$ primer, as described elsewhere (Han et al., 1996).

3.2.4. PCR amplification of IDDMK$_{1,2}$22 from genomic DNA, cytoplasmic RNA, and viral RNA

PCR was carried out on genomic DNA as well as cytoplasmic and viral cDNA. We used 4ul of template for PCR in a buffer containing 50mM KCl, 1.5mM $MgCl_2$, and 10mM Tris-HCl pH 9.0 (Pharmacia Biotech), 1ul of 10mM dNTP mix, 1ul each of 15mM 5' and 3' primer, and 0.5ul of 5000 U/ul Taq polymerase (Pharmacia) in a final reaction volume of 50ul. Three primer sets were used for the first round PCR: i) 5'SAg 5'- CTG CCA AAC CTG AGG AAG AA -3', 3'SAg 5'- CAC CAC ACT ATT GGC CAC AC -3'; ii) U3 5'- AGG TAT TGT CCA AGG TTT CTC C -3', R-poly(A) 5'- TTT TGA GTC CCC TTA GTA TTT ATT -3'; iii) U3 as above, R'- GTA AAG GAT CAA GTG CTG TGC -3'. The primers U3N 5'- GGG CAA TGG AAT GTC TCA GTA -3', and R as above, were used for nested PCR of the U3/R-poly(A) PCR product. Cycle conditions for PCR were as follows: 10 cycles of 94°C for 15sec, 68°C for 30sec with -1.3°C per cycle, and 72°C for 45sec; 30 cycles of 94°C for 15sec, 55°C for 30sec, and 72°C for 45sec. The PCR product was run on a 1% agarose gel and detected by ethidium bromide staining.

3.2.5. Control PCR reactions.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) PCR was run on 4ul of template in 50 ul of a reaction mix containing 50mM KCl, 1.5mM $MgCl_2$, and 100mM Tris-HCl (pH 9.0) (Pharmacia), 1ul 10mM dNTP mix, 1ul of 10mM 5'-GAPDH primer (5'- CGG AGT CAA CGG ATT TGG TCG TAT -3'), 1ul of 10mM 3'-GADPH primer (5'- GTC TTC ACC ACC ATG GAG AAG GCT-3') and 0.5ul of 5000U/ul Taq polymerase (Pharmacia). The cycle conditions for the PCR were as follows: 35 cycles of 94°C for 1min, 55°C for 30sec, 72°C for 2min.

Interleukin-2 (IL-2) intervening sequence 3 (IVS-3) primers were used for the detection of genomic DNA in viral and cytoplasmic RNA isolates. The primer sequences were as follows: IL-2 IVS-3 5' primer 5'- TAG CTC CTT TCA GCA GAG AAG -3'; IL-2 IVS-3 3' primer 5'- GTC TTA TAG GCC TGT TGC CTT -3'. Viral RNA (1.6ul) or cytoplasmic RNA (2.2ul) was added to the same reaction mix used for GADPH PCR with substitution of the appropriate primers. The cycle conditions were as follows: 40 cycles at 94°C for 1min, 55°C for 30s, 72°C for 1min.

3.2.6. Sequencing of the U3/R-poly(A) PCR product

Following PCR, the samples were purified using the QIAquick PCR purification kit (Qiagen) and directly sequenced using the U3 primer.

3.3. RT mediated persistence of EMC-D virus and its role in the pathogenesis of murine IDDM

3.3.1. Mice

Eight to 12 week old DBA/2 male mice were purchased from Jackson Laboratories (Bar Harbor, Maine). All animals were maintained on site at the Animal Resource Centre (ARC) of the University of Calgary under specific pathogen free (SPF) conditions. They were fed autoclaved food and water ad libitum.

3.3.2. Preparation of EMC-D virus

EMC-D virus was cultured in L929 cells grown in RPMI 1640 (Gibco BRL) and plaque assayed as described elsewhere (Yoon et al., 1988). Serial dilutions were prepared in

phosphate buffered saline (PBS) and 100pfu was injected intraperitoneally into metofane anesthetized mice.

3.3.3. Preparation of L929 cell lysate control

An EMC-D virus-free culture of L929 cells was subjected to 3 successive freeze-thaw cycles in an ethanol/dry ice bath. Serial dilutions were prepared and a 100pfu equivalent was injected as in section 3.3.2..

3.3.4. Measurement of blood glucose and diabetes criteria

Animals were bled from the tail vein and 15ul of blood was collected. Blood glucose was measured with a One-Touch Basic glucometer (Lifescan, Burnaby, BC). The mean blood glucose level of 36, L929 cell lysate control infected, DBA/2 male mice was 127 ± 49 mg/dl. A mouse with a blood glucose level greater than 274 mg/dl, i.e. 3 SD greater than the mean, was scored as diabetic.

3.3.5. Standard procedure for isolation of genomic DNA

All tissues intended for genomic DNA isolation were immediately snap frozen and stored in liquid nitrogen after harvesting. Genomic DNA was isolated using the DNeasy Tissue Kit (Qiagen) according the manufacturer's instructions with the following modifications: a 2x volume of proteinase K was used in the initial digestion step; a 60min RNase A treatment at a final concentration of 2 mg/ml was included.

3.3.6. Standard procedure for PCR amplification of an EMC-D VP1 specific sequence

1ug of genomic DNA sample was added to a reaction mixture containing 50mM KCl, 2.5mM $MgCl_2$, and 10mM Tris-HCl pH 9.0 (Pharmacia), 1ul of 10mM dNTP mix, 1ul each of 15mM 5' and 3' primer, and 0.5ul of 5000 U/ul Taq polymerase (Pharmacia) in a final reaction volume of 50ul. Primers specific for EMC-D VP1 were used: 5'EMC-D VP1, 5'- ACA GTT TGA CCC CGC TTA -3'; 3'EMC-D VP1, 5'- AGC TCT CGG GGT CAT GTC TA -3'. Cycle conditions for PCR were as follows: an initial denaturation step of 95°C for 5min was followed by

10 cycles of 95°C for 1min, 67°C for 30sec with -1.3°C per cycle, and 72°C for 45sec; 30 cycles of 95°C for 30sec, 57°C for 30sec, and 72°C for 45sec; 72°C for 7min. PCR products were run on a 1% agarose gel and detected by ethidium bromide staining.

3.3.7. Isolation of EMC-D viral RNA

L929 cells grown in a 75cm^2 flask were infected with EMC-D virus and incubated for 12-24h. Cells harboring viral RNA were harvested by scraping and centrifuged at 1500xg for 5 min. The supernatant was removed and viral RNA was isolated with Trizol reagent (Gibco BRL) according to the manufacturer's instructions. The isolated viral RNA was treated with a final concentration of 2U/ul of DNase I (Boehringer Mannheim) at 37°C for 60 min. The DNA-free viral RNA was then re-isolated by a commercial cleanup protocol using a RNeasy Mini kit (Qiagen).

3.3.8. Sequencing of the EMC-D VP1 primer specific PCR product

PCR products were purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. Purified PCR product was directly sequenced using the 5'EMC-D VP1 primer from section 3.3.6..

3.3.9. Southern blotting of PCR products

PCR products were visualized by gel electrophoresis on 1% agarose and transferred to nylon membrane (Boehringer Mannheim) using a semi-dry transfer apparatus (BioRad). Nylon membrane was hybridized with DIG-labeled probe specific for EMC-D VPI.

Specific probe was generated by digestion of plasmid EMC/Blu containing the VP1 gene with Bgl II and BST XI that cut specifically at position 115 and 592 of the 831bp VP1 gene to yield a 477bp fragment. This fragment was separated on a 1% agarose gel and purified using the QIAquick gel extraction kit (Qiagen). The fragment was labeled with the DIG DNA Labeling and Detection Kit according to the manufacturer's instructions.

DIG-labeled probe was hybridized to transferred membrane and southern blotting was carried out as outlined in the Guide to Non-radioactive Research: v.1 (Boehringer Mannheim). The labeled blot was developed with CSPD (Tropix, Bedford, MA).

3.4. RT mediated persistence of CB4 virus and its role in the pathogenesis of human IDDM

3.4.1. Tissue collection

Pancreatic tissue was collected from a recently deceased IDDM patient. Immediately following transplantation protocols the pancreas was harvested by Dr. Cynthia Trevanen, staff pathologist at the Alberta Childrens Hospital (ACH). The organ was divided into head and tail sub-portions that were either snap frozen immediately for subsequent isolation of genomic DNA or transferred to 10% buffered formalin for histological examination.

3.4.2. Histological analysis of human pancreas

Pancreatic tissue fragments were fixed in 10% buffered formalin for 12 hours and embedded in paraffin wax. 3um thick sections were cut and stained with hematoxylin and eosin. Specific stains for insulin and CD8+ T-cells were also carried out. Guinea pig α-human α-insulin antibody purchased from DAKO (Missisauga, ON). Treated slides were bound with biotinylated α-guinea pig IgG and developed with the ABC peroxidase kit (Vector). Rabbit α-human α-CD8 antibody was purchased from DAKO (Missisauga, ON). Sections were pretreated with 0.05% trypsin at 37°C for 5min and boiled in 10mM EDTA pH 8.0 for 5min. Slides were then treated with rabbit α-human α-CD8 antibody and developed with the Histostain Plus Detection Kit (Zymed).

3.4.3. Standard procedure for isolation of genomic DNA

Genomic DNA was isolated as in section 3.3.4.

3.4.4. Standard procedure for PCR amplification of a CB4 VP1 primer specific sequence

PCR was carried out as in section 3.3.6. with CB4 VP1 specific primers: 5'CB4 VP1, 5'- CGG ATC CGG GCC AAC AGA GGA ATC TGT GGA GCG -3': 3'CB4 VP1, 5'- CGA ATT CAA TGA GGT CCA GTG GTT ACC AGA CTT GC -3'.

4. Results and Discussion

4.1 A unique endogenous retrovirus in NOD mice

4.1.1. NOD mouse β-cell derived λgt11 cDNA library

The NOD mouse is an inbred animal model for IDDM that was developed in the late 1970's from the ICR mouse. It is characterized by abrupt onset of overt diabetes, insulin dependence, tendency to keto-acidosis without treatment, and massive infiltration of lymphocytes in and around the pancreatic islets (Makino and Tochino, 1978; Tochino, 1979). The incidence, time of onset, and preponderance of the disease in females differs from colony to colony. In general, insulitis is seen in almost all animals, with a greater percentage of females developing overt diabetes (~70-80% of females vs. 10-20% of males). Diabetes usually manifests between 11 and 28 weeks of age (Kolb, 1987). The source cell line used for this study, mouse insulinoma 6N8a (MIN6N8a), was derived from NOD β-cells (Kasuga et al., 1993). mRNA was isolated MIN6N8a and a cDNA library was constructed using a phage λgt11 vector system. Nine positive clones were selected from $2x10^5$ λgt11 plaques in our MIN6N8a cDNA library.

4.1.2. Sequence analysis of NOD sera reactive λgt11 clones

The nucleotide sequence of the 5′ end of each positive clone was determined. A search of the Genbank database revealed that 6 our 9 positive clones had a high degree of homology with published endogenous murine leukemia retroviral envelope protein sequences. Detailed analysis of the nucleotide sequence of our 6 positive clones showed that each of these clones represents a different region of the same retroviral envelope gene. Further analysis was carried out on the smallest clone (3-2). Not including the poly A region, the 3-2 clone was a 1010 nucleotide fragment (Figure 1). Within the open reading frame (ORF) this clone possessed only a 1 to 3 nucleotide difference (99.4% to 99.8% homology) with published endogenous murine leukemia retroviral envelope gene sequences (Table 5). It was therefore concluded that clone 3-2 represented a portion of the mouse endogenous leukemia proviral envelope gene. Figure 1 shows the full nucleotide sequence of clone 3-2 and its open reading frame. Only a single nucleotide

Figure 1. Nucleotide and amino acid sequence of clone 3-2. The nucleotide sequence of clone 3-2 was determined by cycle DNA sequencing. The amino acid sequence was deduced from the determined nucleotide sequence. Only a single nucleotide difference was present in our 3-2 clone when homology with mouse endogenous leukemia virus envelope protein was examined. Our clone has a T base at position 497 while there is a C base in the published sequence. The location of the nucleotide in question is marked (_).

```
  1 GGGACTACCG CCCTAGTGGC CACTCAGCAG TTCCAACAAC TCCAGGCTGC CATGCACGAT   60
  1  G  T  T   A  L  V  A   T  Q  Q   F  Q  Q   L  Q  A  A   M  H  D   20

 61 GACCTTAAAG AAGTTGAAAA GTCCATCACT AATCTAGAAA AATCTTTGAC CTCCTTGTCC  120
 21  D  L  K   E  V  E  K   S  I  T   N  L  E   K  S  L  T   S  L  S   40

121 GAAGTAGTGT TACAGAATCG TAGAGGCCTA GATCTACTAT TCCTAAAAGA GGGAGGTTTG  180
 41  E  V  V   L  Q  N  R   R  G  L   D  L  L   F  L  K  E   G  G  L   60

181 TGTGCTGCCT TAAAAGAAGA ATGCTGTTTC TATGCCGACC ACACAGGATT GGTACGGGAT  240
 61  C  A  A   L  K  E  E   C  C  F   Y  A  D   H  T  G  L   V  R  D   80

241 AGCATGGCCA AACTTAGAGA AAGATTGAGT CAGAGACAAA AGCTCTTTGA ATCCCAACAA  300
 81  S  M  A   K  L  R  E   R  L  S   Q  R  Q   K  L  F  E   S  Q  Q  100

301 GGGTGGTTTG AAGGGCTGTT TAATAAGTCC CCTTGGTTCA CCACCCTGAT ATCCACCATC  360
101  G  W  F   E  G  L  F   N  K  S   P  W  F   T  T  L  I   S  T  I  120

361 ATGGGTCCCC TGATAATCCT CTTGTTAATT TTACTCTTTG GGCCTTGTAT TCTCAATCGC  420
121  M  G  P   L  I  I  L   L  L  I   L  L  F   G  P  C  I   L  N  R  140

421 CTGGTCCAGT TTATCAAAGA CAGGATTTCG GTAGTGCAGG CCCTGGTTCT GACTCAACAA  480
141  L  V  Q   F  I  K  D   R  I  S   V  V  Q   A  L  V  L   T  Q  Q  160

                  (C)
481 TATCATCAAC TTAAGATAAT AGAAGATTGT AAATCACGTG AATAAAAGAT TTTATTCAGT  540
161  Y  H  Q   L  K  I  I   E  D  C   K  S  R   E  *                  175
                  (T)

541 TTACAGAAAG AGGGGGGAAT GAAAGACCCC TTCATAAGGC TTAGCCAGCT AACTGCAGTA  600
175                                                                   175

601 ACGCATTTTG CAAGGCATGG GAAAATACCA GAGCTGATGT TCTCAGAAAA ACAAGAACAA  660
175                                                                   175

661 GGAAGTACAG AGAGGCTGGA AAGTACCGGG ACTAGGGCAA ACAGGATATC TGTGGTCAAG  720
175                                                                   175

721 CACTAGGGCC CCGGCCCAGG GCCAAGAACA GATGGTCCCC AGAAATAGCT AAAACAACAA  780
175                                                                   175

781 CAGTTTCAAG AGACCCAGAA ACTGTCTCAA GGTTCCCCAG ATGACCGGGG ATCGGCCCCA  840
175                                                                   175

841 AGCCTCATTT AAACTAACCA ATCAGCTCGC TTCTCGCTTC TGTACCCGCG CTTATTGCTG  900
175                                                                   175

901 CCCAGCTCTA TAAAAAGGGT AAGAACCCCA CACTCGGCGC GCCAGTCCTT CGATAGACTG  960
175                                                                   175

961 AGTCGCCCGG GTACCCGTGT ATCCAATAAA GCCTTTTGCT GTTGCATCCG AAAAAAAAAA 1020
175                                                                   175
```

Table 5 List of genes having homology with λgt11 clone 3-2

No.	Name	Accession No.	Homology (%/size*)
1	Mouse endogenous murine leukemia proviral env region DNA	M21028	99.8/525
2	Mouse mRNA for mouse melanoma antigen	D10049	99.6/525
3	Mouse (strain C3H) endogenous C3H/He murine leukemia proviral pol and env region DNA	M19005	99.6/525
4	M.musculus env mRNA from SV-40 transformed cells	X62670	99.4/525
5	Mouse endogenous ecotropic murine leukemia proviral locus 3(Emv-3) envelope glycoprotein(env) gene	L37057	99.4/525
6	Mouse endogenous MuLV ecotropic loci putative pol genes, gag and env genes	M87550	98.8/512
7	Mouse endogenous ecotropic proviral gene, env gene region	M11751	100.0/154

* Accession number: the accession number of gene in Genbank database.

difference was detected with the published sequence of greatest homology, the mouse endogenous leukemia proviral envelope proviral DNA (Genbank accession no. M21028). The sequence of clone 3-2 covers the greater portion of the transmembrane (TM) region, including the leucine zipper and transmembrane anchor regions, of the envelope protein (Figure 3).

4.1.3. Expression of λgt11 clone 3-2 in E.coli

The retroviral sequence from clone 3-2 was subcloned into the pET30b expression vector, which was then transformed into E.coli BL21. The recombinant retroviral protein was expressed as a six histidine/S protein fusion protein, with a molecular weight of 27kD as visualized by SDS-PAGE (Figure 2). The size of this protein coincides with the theoretical estimation of molecular weight (25.3 kD) based on the deduced amino acid sequence of the 230 amino acid translated reading frame which includes 175 amino acids of retroviral envelope protein (Figure 1), 50 amino acids of fusion protein, and 5 amino acids from the Eco RI linker. The majority of the expressed recombinant protein was found in the insoluble fraction (Figure 2, Lane

Figure 2. Expression of purified recombinant retroviral envelope protein. The retroviral envelope sequence from λgt11 clone 3-2 was subcloned into the expression vector pET30b. The new construct was transformed into E.coli BL21 and the recombinant 3-2 protein was expressed via isopropyl-1-thio-β-D-galactoside (IPTG) induction. After purification by Ni-NTA affinity chromatography the recombinant 3-2 protein was visualized by SDS-PAGE. Lane 1: Whole extracts of recombinant E.coli. Lane 2: Insoluble fraction of recombinant E.coli extract. Lane 3: Purified recombinant 3-2 protein. The arrow indicates the expressed recombinant 3-2 envelope protein.

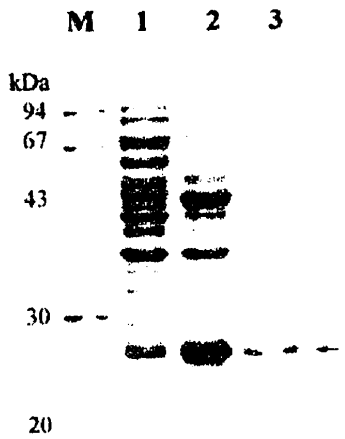
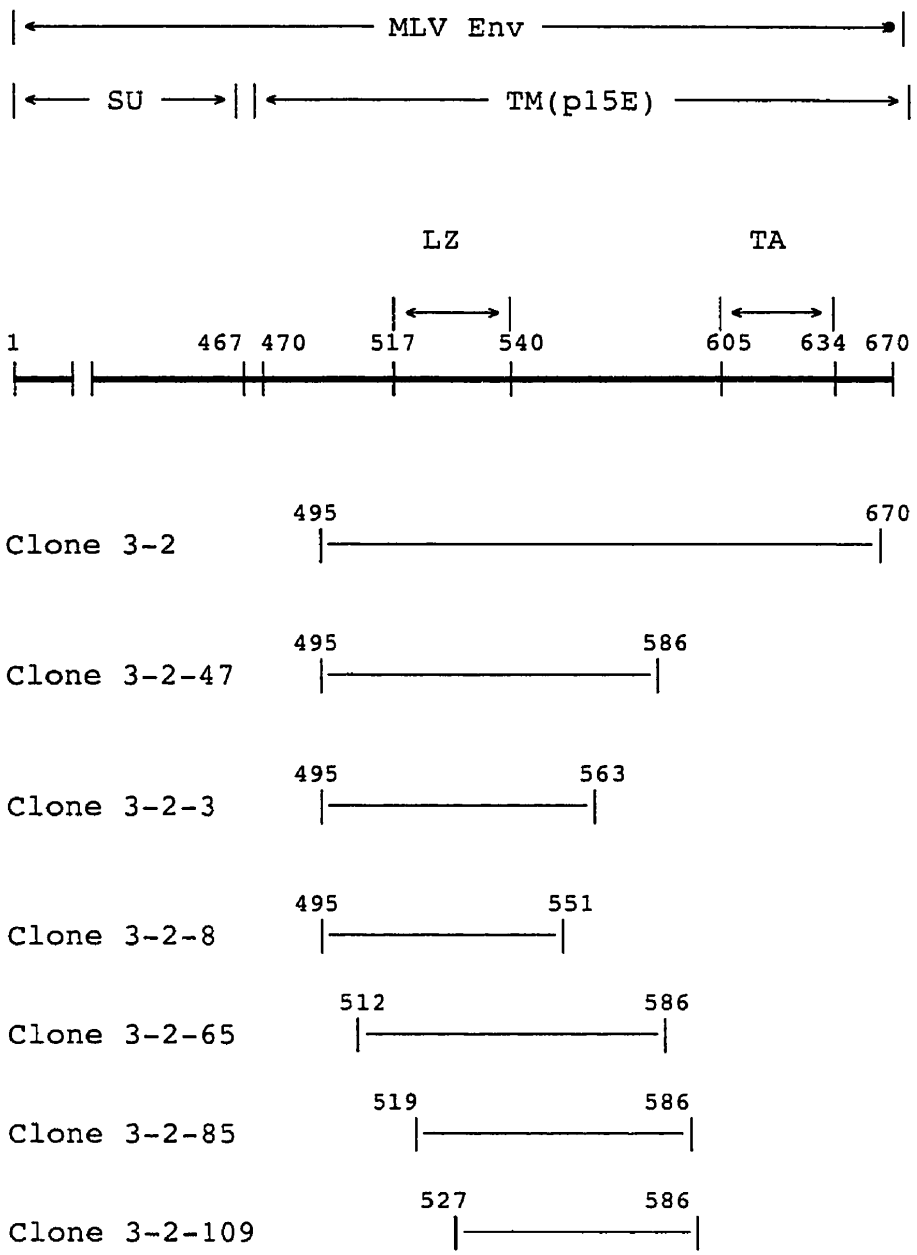

M
1
2
3
kDa
94
67
43
30
20

Figure 3. λgt11 clone 3-2 deletion mutants. Schematic diagram of the murine leukemia virus envelope protein and the predicted location of the recombinant 3-2 envelope protein and its deletion mutants. MLV: murine leukemia virus. SU: surface region of the retroviral envelope protein. TM: transmembrane region of the envelope protein. LZ: leucine zipper domain of the TM region. TA: transmembrane anchor domain of the TM region.


MLV Env
SU
TM(p15E)
LZ
TA
1
467
470
517
540
605
634
670
Clone 3-2
495
670
Clone 3-2-47
495
586
Clone 3-2-3
495
563
Clone 3-2-8
495
551
Clone 3-2-65
512
586
Clone 3-2-85
519
586
Clone 3-2-109
527
586

2), indicating that this protein likely exists in the cell as an inclusion body. The relative yield of our expressed protein was low (<3%) in comparison to the most highly expressed proteins in the cell. We purified our recombinant protein using a Ni-NTA affinity purification system. Our recombinant protein was purified using a Ni-NTA affinity purification system and utilized for subsequent T-cell proliferation assay (see section 4.1.5.)

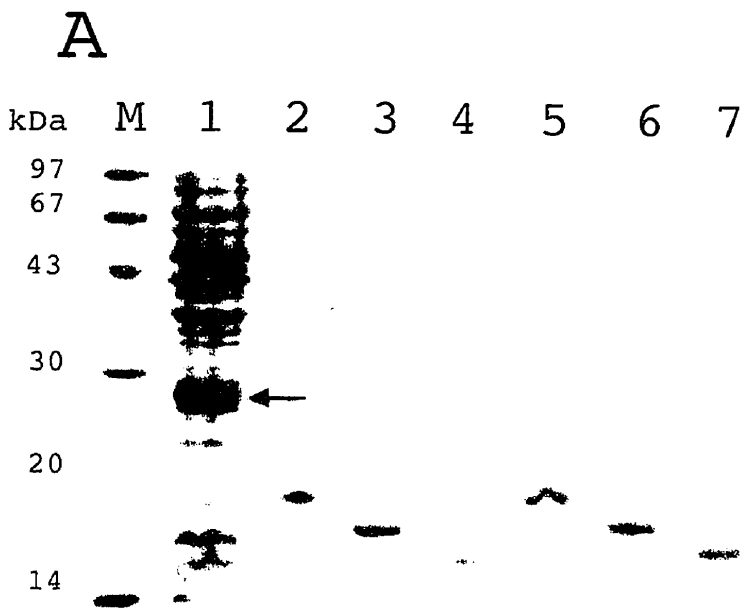
4.1.4. Generation of recombinant retroviral envelope deletion mutants

Deletion mutants were generated from our original 3-2 clone by Bal 31 degradation of the 3′ end of the insert sequence and PCR amplification from the 5′ end. The resulting gene fragments were subcloned into the pET30b expression vector and transformed into E.coli BL21 as in section 4.1.3. (Figure 3).

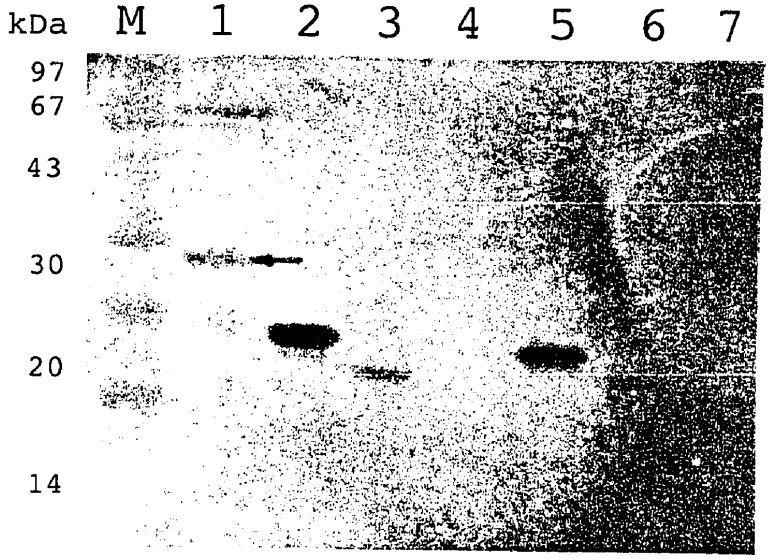
4.1.5. Epitope mapping of recombinant retroviral envelope protein

The expressed proteins from our original 3-2 clone and the deletion mutants generated in section 4.1.4. were visualized by SDS-PAGE as in section 4.1.3. (Figure 4A). The yield of expression of those deletion mutants lacking the hydrophobic transmembrane domain was elevated compared to our original clone 3-2. In order to investigate possible humoral reactivity of both the original recombinant retroviral protein and those generated from our deletion mutants, the SDS-PAGE visualized proteins were analyzed by western blotting (Figure 4B) using prediabetic NOD sera. The low level of reactivity of clone 3-2 (Figure 4B, Lane 2) was likely due to the meager amount of available protein that was loaded in the original gel. The low levels of clone 3-2-3 (Figure 4B, Lane 4) and clone 3-2-85 (Figure 4B, Lane 7) were likely due to the loss of a large portion of the antigenic epitope. The amount of protein loaded in the case of these clones was similar to clone 3-2-47 (Figure 4B, Lane 3) and clone 3-2-65 (Figure 4B, Lane 6). Based on the nucleotide sequence of positive (highly expressed) and negative (low levels of expression) deletion

Figure 4. Expression and immune reactivity of clone 3-2 and clone 3-2 deletion mutants. The insert from λgt11 clone 3-2 was sequentially deleted by Bal31 digestion and/or PCR amplification with specific 5′ primers. The truncated 3-2 sequences were subcloned into the pET30b expression vector. (a) E.coli extracts of the transformed construct gene products were visualized by coomassie blue staining of SDS-PAGE. (b) Western blotting of SDS-PAGE with prediabetic NOD sera. Lane 1: Whole extracts of recombinant 3-2 E.coli. Lane 2: Clone 3-2-47. Lane 3: Clone 3-2-3. Lane 4: Clone 3-2-8. Lane 5: Clone 3-2-65. Lane 6: Clone 3-2-85. Lane 7: Clone 3-2-109.


A
kDa
M
1
2
3
4
5
6
7
97
67
43
30
20
14

B

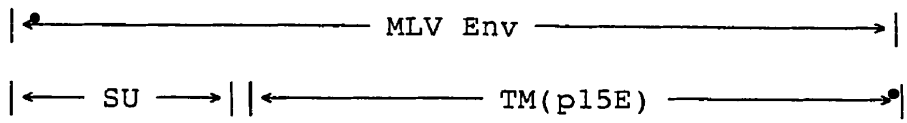


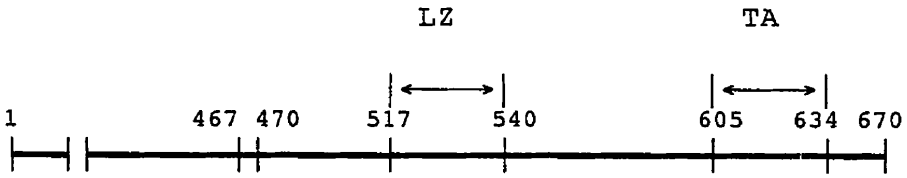
mutants the target epitopes were localized to a leucine zipper motif in the transmembrane region of our putative retroviral envelope protein (Figure 5).

4.1.6. Comparison of humoral immunity to retroviral envelope protein in NOD vs. non-diabetic mice

The extent of the humoral immune response against our retroviral envelope protein was determined in NOD mice and a variety of non-diabetes prone strains. A 100-fold dilution of serum from individual mice was used to perform western blotting. Approximately 45% of prediabetic and/or acute diabetic NOD mice along with 50% of non-obese non-diabetic (NON) mice exhibited reactivity against our retroviral envelope protein. Other strains, including Balb/c, ICR (the parent strain of both NOD and NON), C57BL/6, SJL/J and the T-cell and B-cell deficient severe combined immunodeficiency NOD (NOD-SCID) did not exhibit antibody reactivity to our retroviral envelope protein (Table 6).

Figure 5. Mapping of the immune reactivity of clone 3-2 deletion mutants. Schematic diagram of the murine leukemia virus envelope protein, recombinant 3-2 envelope protein, and 3-2 deletion mutant proteins with correlated immune reactivity as determined by western blotting with prediabetic NOD sera. MLV: murine leukemia virus. SU: surface region of the retroviral envelope protein. TM: transmembrane region of the envelope protein. LZ: leucine zipper domain of the TM region. TA: transmembrane anchor domain of the TM region.





		Immune reactivity
Clone 3-2	495–670	+
Clone 3-2-47	495–586	+
Clone 3-2-3	495–563	+/-
Clone 3-2-8	495–551	-
Clone 3-2-65	512–586	+
Clone 3-2-85	519–586	+/-
Clone 3-2-109	527–586	-

Table 6 Humoral immunity against clone 3-2 encoded retroviral envelope protein

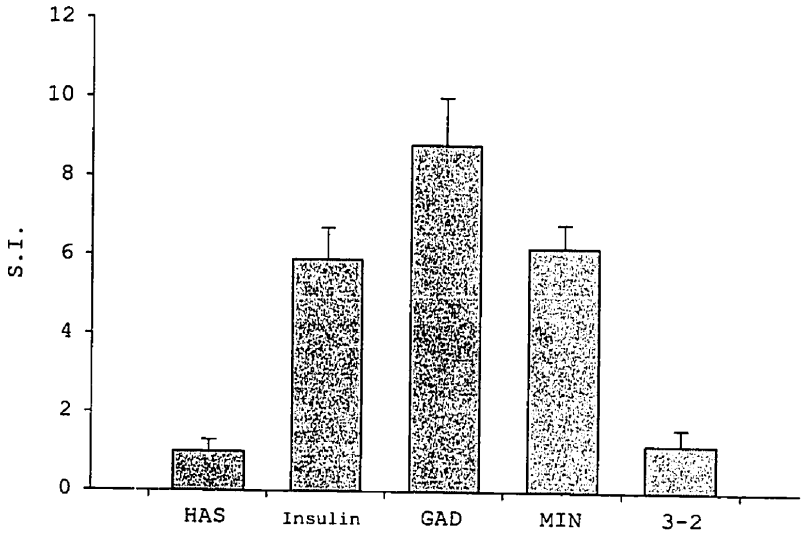
Mouse Strain	Number of positive mice/ Number of test mice	prevalence of envelope-positive autoantibody (%)
NOD-SCID	0/3	0
NOD(P.D)	11/24	45.8
NOD(A.D)	5/11	45.4
NON	2/4	50
Balb/c	0/6	0
ICR	0/6	0
C57BL/6	0/4	0
SJL/J	0/4	0

The immune reactivity was determined by western blotting of purified recombinant 3-2 retroviral envelope protein with 100 fold diluted individual serum. Alkaline phosphatase-conjugated α-mouse IgG was used as secondary antibody. The color was developed by addition of BCIP/NBT. The positive activity was determined by appearance of color. P.D. = Pre-Diabetic; A.D. = Acutely Diabetic.

4.1.7. Cell-mediated immunity against purified retroviral envelope protein in NOD mice

IDDM is a T-cell mediated autoimmune disease so to test whether our retroviral envelope protein was capable of triggering a MHC Class II linked cell-mediated immune response a T-cell proliferation assay was carried out. Relevance of the exogenous or endocytic pathway of antigen presentation was examined by mixing T-cells and antigen presenting cells (APC's) from NOD mice in a reaction with purified recombinant retroviral envelope protein (from section 4.1.3.). After 3 days the proliferation of retroviral envelope protein specific T-cells was quantified by measurement of the incorporated ^{3}H-thymidine. Cell-mediated immunity, as determined by this method, was low compared to the level of proliferation seen in response to known IDDM autoantigens such as insulin and GAD. The level of proliferation in T-cells isolated from prediabetic NOD mice in response to our retroviral envelope protein was similar to that seen in response to human serum albumin (Figure 6).

Figure 6. Cell-mediated immunity against clone 3-2 encoded retroviral envelope protein. The proliferation of splenic T-cells from NOD mice in response to various autoantigens was determined. γ-irradiated splenocytes were used as APC's. A T-cell enriched fraction was column isolated and mixed with APC's. Proliferation of antigen specific T-cells was determined by measurement of incorporated ^{3}H-thymidine. The stimulation index is the ratio between the sample counts per minute (cpm) value and the cpm of HSA negative control in the same assay. HSA: human serum albumin. GAD: glutamate decarboxylase. MIN: whole protein extract of the mouse insulinoma 6N8a (MIN6N8a) cell line.


12
10
8
6
4
2
0
S.I.
HAS
Insulin
GAD
MIN
3-2

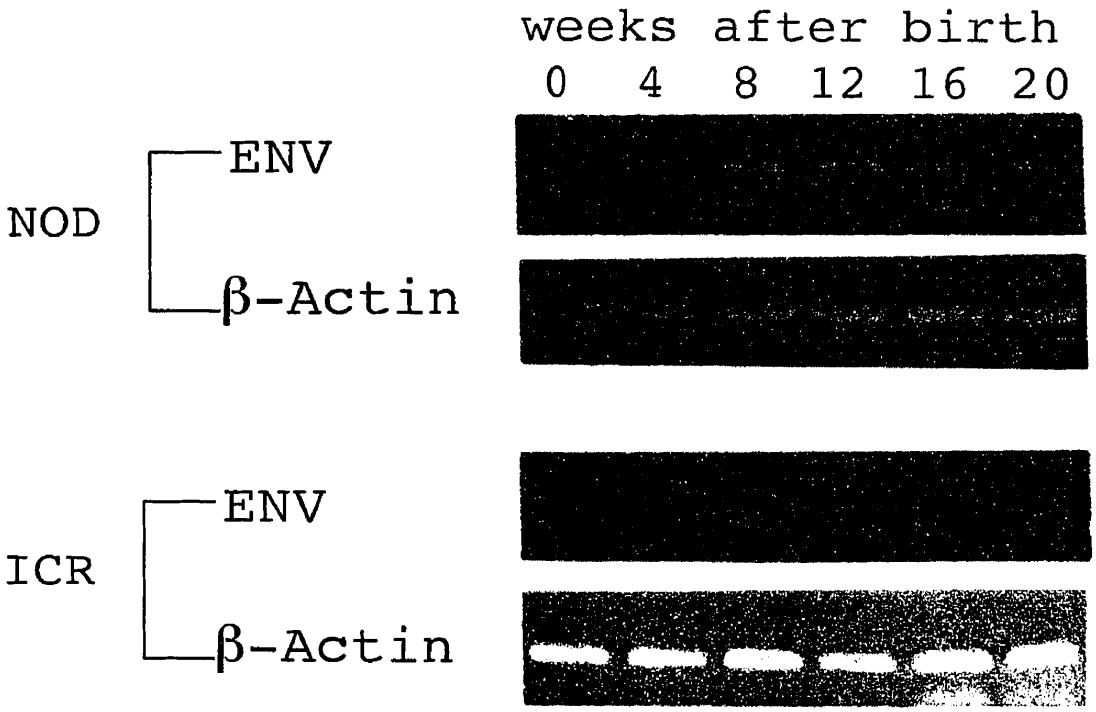
4.1.8. Age dependent expression of endogenous murine leukemia virus envelope gene in NOD mice

Expression of endogenous murine leukemia virus envelope gene was examined via reverse transcriptase PCR (RT-PCR). Gene transcription was elevated in NOD mice at 8 and 12 weeks of age (p.i.), coincidentally the age at which maximal mononuclear cell infiltration of the pancreatic islets occurs in NOD mice. The level of gene expression was decreased at 16 and 20 weeks of age, the age when clinical diabetes is usually observed. Expression was not significant in control ICR mice at any age (Figure 7).

4.1.9. Discussion

This study was initially directed at the identification and characterization of new autoantigens associated with IDDM. Nine positive clones were selected from a MIN6N8a cDNA library. Six of the 9 positive clones were identified as regions of a single endogenous murine leukemia retroviral envelope gene. The 3 remaining clones were sequences lacking significant homology to any published genes. Genes for established autoantigens such as

Figure 7. Age dependent expression of endogenous murine leukemia virus envelope gene. Gene expression of clone 3-2 was analyzed by RT-PCR. cDNA generated from pancreatic total RNA of n=5-10 NOD mice were amplified with primers specific for the endogenous murine leukemia virus envelope gene. RT-PCR detection of mouse β-actin was performed as a control. The amplified products were visualized by electrophoresis on a 1% ethidium bromide stained agarose gel.


weeks after birth
0 4 8 12 16 20
NOD
ENV
β-Actin
ICR
ENV
β-Actin

insulin, GAD, or IA-2 were not selected. In the case of insulin and GAD this is not surprising as it has already been established that both show weak reactivity to NOD mouse serum (Kikutani and Makino, 1992). The fact that two thirds of the positive clones from our MIN6N8a cDNA library represent portions of the same retroviral envelope gene likely reflects high levels of retroviral mRNA expression in MIN6N8a cells. It has been reported that cell transformation by SV40 T antigen can induce abnormal expression of endogenous proviral genes (Feuchter and Mager, 1992). As the MIN6N8a cell line was generated by transformation by the SV40 T antigen (Kasuga et al., 1993) it should be recognized that endogenous retroviral genes are abnormally expressed in MIN6N8a cells. It would have been preferable to employ a primary culture of NOD β-cells in place of MIN6N8a cells but it was determined experimentally that the isolation of the necessary number of islets was logistically unfeasible and that inherent heterogeneity in the primary culture might contribute unreasonable bias.

Epitope mapping using deletion mutants revealed that the target epitopes were located in a leucine zipper motif in the transmembrane region of our putative retroviral

envelope protein (Figure 5). It is likely that recognition resides within the extracellular portion of the retroviral transmembrane p15E protein within the target epitope spanning leucine zipper motif and the transmembrane anchor region (Ramsdale et al., 1996). It is thus probable that if the same retroviral envelope protein were expressed in the β-cells of NOD mice as in MIN6N8a cells it would be recognized on the cell surface by NOD autoantibodies. It has been postulated that aberrant expression of retroviral envelope proteins on the surface of the β-cell, induced by hormonal factors or cytokine release due to subsequent viral infections, could act as a triggering autoantigen in a β-cell specific autoimmune response (Nakagawa and Harrison, 1996).

It is apparent that NOD autoantibodies are capable of detecting a linear epitope as the denatured protein encoded by clone 3-2 was recognized by western blotting. Since deletion at the 5′ or 3′ end of clone 3-2 lowered the strength of immune reactivity to the resulting proteins it was concluded that the entire 55 amino acid segment encoded by clone 3-2 could be recognized by NOD autoantibodies. A 55 amino acid sequence is too long to represent a single

target epitope and so the immune reactivity that we were able to detect does not represent a monoclonal antibody. The fact still remains that though polyclonal autoantibodies in NOD sera are reactive to the clone 3-2 protein, the deletion of several amino acids from either end of the protein greatly decreased reactivity. It is thus probable that most of the reactive antibodies recognize a relatively short region of the protein. This epitope or epitopes are novel and differ from previously reported retroviral antigen epitope(s) (Serreze et al., 1988).

Autoantibodies to our retroviral envelope protein exist in NOD ad NON mice but diabetes resistant strains such as Balb/c, ICR, C57BL/6, and SJL/J showed no such reactivity. The NON strain is a non-diabetic strain, albeit with weak insulitis, and shares a significant portion of the NOD genetic background. It is thus likely that both a NOD genetic background and some initial insult to the pancreatic β-cell are required for the induction of humoral immunity against our retroviral envelope protein. The prevalence of retroviral envelope specific autoantibody was similar in both prediabetic and acutely diabetic NOD mice (Table 6). This is in contrast to the report that reactivity to other autoantibodies decreases after onset of

diabetes (Ko et al., 1991). Although humoral immunity in NOD mice was distinct, cell-mediated immunity against our retroviral envelope protein was weak when examined by a standard T-cell proliferation assay (Figure 6).

RT-PCR analysis of endogenous MuLV envelope gene expression revealed a pattern consistent with a scenario in which retroviral expression might play a role in IDDM pathogenesis. It is known that maximal mononuclear cell infiltration into the pancreatic islets of NOD mice occurs between 8 and 12 weeks of age. Thus the severity of insulitis was shown to be consistent with the observed increase in MuLV envelope gene expression levels as detected in this study.

IDDM is believed to be a T-cell mediated autoimmune diseases. The absence of a β-cell specific cell-mediated immune response in NOD mice suggests that the retroviral envelope protein encoded by the 3-2 clone is not a critical autoantigen in the destruction of pancreatic β-cells and IDDM pathogenesis. A role for humoral reactivity cannot be ascribed in this model beyond that of an epiphenomenon subsequent to β-cell destruction. It has been reported that a short peptide sequence, CKS-17, which maps to a site

within the 3-2 retroviral envelope protein, possesses immunosuppressive activity (Haraguchi et al., 1997). It is thus possible that our 3-2 retroviral envelope protein may retard the progression of cell-mediated destruction of pancreatic β-cells in NOD mice rather than accelerating IDDM onset. The long duration of the prediabetic phase is not contradictory to a counterbalancing scenario in which the protective effects of an immunosuppressive activity attributed to the CKS-17 peptide must be overcome before significant destruction of NOD pancreatic β-cells can take place. It remains for this hypothesis to be tested experimentally.

4.2. The role of the retroviral sequence IDDMK$_{1,2}$22 in the pathogenesis of human IDDM

It has been reported previously that a preponderance of islet-infiltrating T-cells bearing a particular T-cell receptor β-chain segment (Vβ7) was attributable to the presence of a superantigen (Conrad et al., 1994). It was not known, however, whether the superantigen was encoded by a bacterium or an endogenous retrovirus. A subsequent study

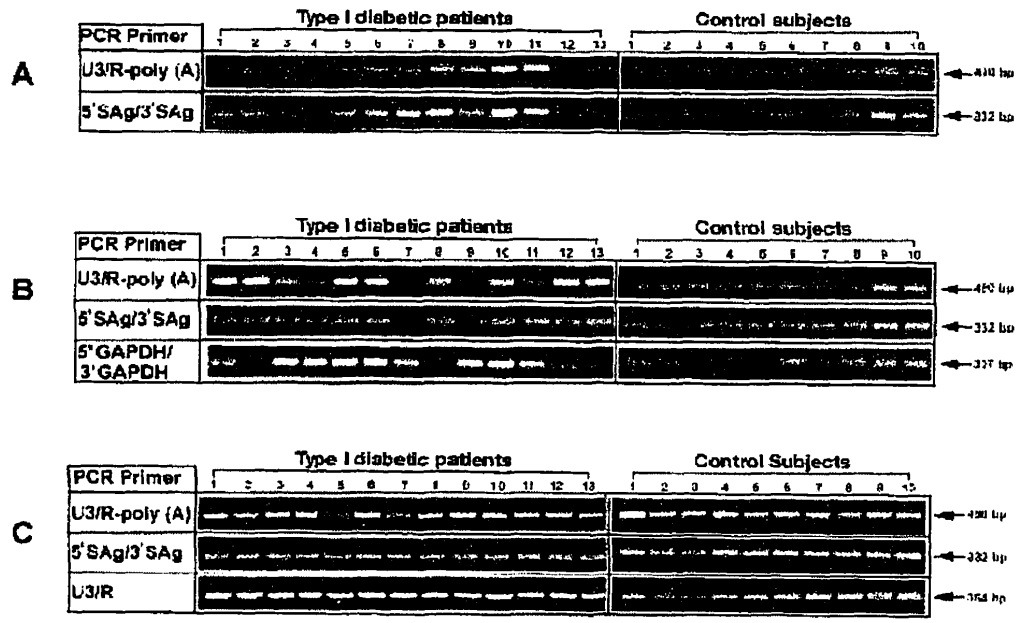
showed that the superantigen was encoded by a novel human endogenous retroviral gene designated IDDMK$_{1,2}$22 (Conrad et al., 1997). IDDMK$_{1,2}$22 was thought to be a novel member of the mouse mammary tumour virus (MMTV)-related family of human endogenous retrovirus (HERV)-K. This newly identified retrovirus was reported to be expressed in the plasma of recent onset IDDM patients but not in that of non-diabetic control subjects. The report that a novel human endogenous retroviral gene encoding a superantigen was found exclusively in IDDM patients was extremely intriguing. Therefore, we attempted to confirm the specificity of the selective expression of IDDMK$_{1,2}$22 in the plasma and peripheral lymphocytes of IDDM patients (Kim et al., 1999)

4.2.1. Detection of the IDDMK$_{1,2}$22 sequence in human plasma

A very attractive hypothesis for the involvement of an endogenous retrovirus in T-cell-mediated autoimmune type I diabetes is that polyclonal activation of a Vβ-restricted T-cell subset, triggered by the expression of an endogenous retroviral superantigen in MHC class II positive antigen presenting cells, initiates the destruction of pancreatic

beta cells. Conrad et al. identified a novel human endogenous retroviral gene, IDDMK$_{1,2}$22, encoding a superantigen in plasma from 10 recent onset IDDM patients and 10 age-matched control subjects. IDDMK$_{1,2}$22 was found only in the plasma of IDDM patients and not in that of the control subjects. This finding was very intriguing and was potentially invaluable for our understanding of the initiation of autoimmune IDDM and for early prediction of the disease if, in fact, the results were reproducible. Therefore, we carefully examined the expression of IDDMK$_{1,2}$22 transcripts in the plasma of recent onset IDDM patients and healthy control subjects by RNA specific PCR using the U3/R-poly(A) primer pair specific for IDDMK$_{1,2}$22. In contrast to the aforementioned study we detected PCR amplified product uniformly in all plasma viral RNA samples tested (Figure 8A). To verify the specificity of the U3/R-poly(A) PCR product, we carried out nested PCR using the U3N/R primer set. We also detected the same PCR product in all the samples tested (data not shown). We found no difference in the detection of the U3/R-poly(A) PCR product between IDDM patients and non-diabetic control subjects. To determine whether there was any difference in the detection

Figure 8. Retroviral signal detected in plasma viral RNA from both IDDM patients and healthy control subjects. (A) Viral RNA was reverse transcribed using the R-poly(A) primer in place of oligo(dT). The amplified product was detected in all samples regardless of whether U3/R-poly(A) (490bp product) or 5'SAg/3'SAg (332bp product) primers were used for PCR. (B) A retroviral signal was detected in the cytoplasmic RNA from both IDDM patients and normal control subjects. Cytoplasmic RNA was reverse transcribed using oligo(dT). The amplified product was detected in all samples using the U3/R-poly(A) primer set (490bp product) or 5'SAg/3'SAg primer set (332bp product) for PCR. The quality of cytoplasmic RNA isolation and reverse transcription was confirmed using primers specific for GAPDH for PCR amplification (307bp product). (C) The U3/R-poly(A) primer set is capable of amplifying the appropriate product from genomic DNA. As expected the 5'SAg/3'SAg and U3/R primer sets amplified 332bp and 354bp PCR products, respectively. The U3/R-poly(A) primer set amplified a 490bp PCR product, contrary to previously published findings (Conrad et al., 1997)


A
PCR Primer
Type I diabetic patients
Control subjects
U3/R-poly (A)
5'SAg/3'SAg
B
PCR Primer
Type I diabetic patients
Control subjects
U3/R-poly (A)
5'SAg/3'SAg
5'GAPDH/
3'GAPDH
C
PCR Primer
Type I diabetic patients
Control Subjects
U3/R-poly (A)
5'SAg/3'SAg
U3/R

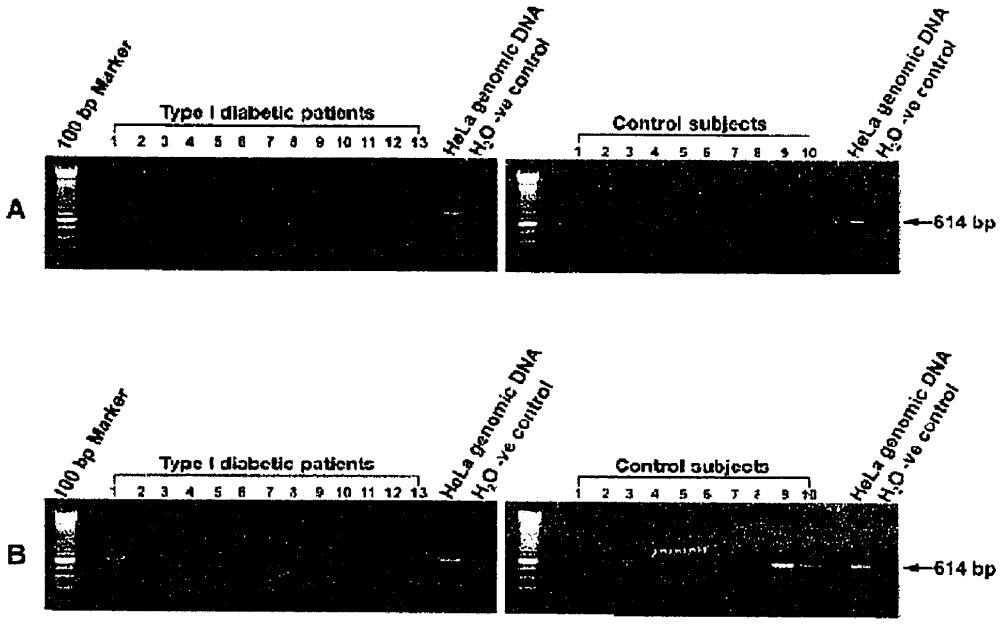
of the endogenous retroviral sequence encoding the superantigen region which is specific for IDDMK$_{1,2}$22 between IDDM patients and control subjects, we designed primers (5'SAg and 3'SAg) from the published sequence, amplified plasma cDNA using the newly designed primer pair, and examined the presence of the amplified products. We detected a 332 base-pair sized amplified product uniformly in all the tested samples (Figure 8A).In addition to plasma samples, we also examined the expression of IDDMK$_{1,2}$22 in the PBMC from diabetic patients and control subjects by RT-PCR using the U3/R-poly(A) primer pair and the 5'SAg/3'SAg primer pair. We obtained the same results as with the plasma samples. The PCR product was detected uniformly in the tested samples (Figure 8B). Conrad et al. showed that the U3/R-poly(A) primer set amplified only the retroviral RNA transcript, but not genomic DNA, while the U3/R primer set amplified genomic DNA. To determine whether the U3/R-poly(A) primer set does not amplify genomic DNA, we did PCR on genomic DNA isolated from PBMC using the U3/R-poly(A), U3/R, and 5'SAg/3'SAg primer sets. We detected a PCR product of the expected size in all samples tested. This result suggests that the U3/R-poly(A) primer set

efficiently amplified the same PCR product from both genomic DNA and RNA (Figure 8C).

4.2.2. Screening for genomic DNA in human plasma viral RNA isolates

It is quite possible that genomic DNA could contaminate the plasma viral RNA preparation and that the contaminating genomic DNA may be amplified in the tested samples. To determine whether there was any DNA contamination in our plasma viral RNA and PBMC RNA preparations, we did PCR of plasma viral RNA and PBMC RNA (without reverse transcription) using IL-2 intron primers (IL-2 IVS-3 5′ and IL-2 IVS-3 3′). We did not detect any PCR product from the plasma viral RNA preparation, but we did detect a PCR product in 8 of 23 PBMC RNA samples (Figure 9A and 9B). This result indicates that there was no genomic DNA contamination in the plasma viral RNA preparation but that some of the PBMC RNA samples did have genomic DNA contamination. There was, however, no difference in the detection of the amplified PCR product (retroviral RNA transcripts) from the PBMC samples (Figure 8B), regardless of the genomic DNA contamination. Thus, the

Figure 9. No genomic DNA detected in the plasma viral RNA isolate from either IDDM patients or healthy control subjects. (A) Plasma viral RNA; IL-2 intron 3 primers did not detect genomic DNA in the non-reverse transcribed viral RNA. (B) Lymphocyte cytoplasmic RNA; IL-2 intron 3 primers detected genomic DNA in the non-reverse transcribed cytoplasmic RNA from 1 of 13 Type I diabetic patients and 7 of 10 healthy control subjects. As a positive control, HeLa genomic DNA was amplified using the same primers. As a negative control, H_2O without template was amplified using the same primers


A
100 bp Marker
Type I diabetic patients
1 2 3 4 5 6 7 8 9 10 11 12 13
HeLa genomic DNA
H2O -ve control
Control subjects
1 2 3 4 5 6 7 8 9 10
HeLa genomic DNA
H2O -ve control
614 bp
B
100 bp Marker
Type I diabetic patients
1 2 3 4 5 6 7 8 9 10 11 12 13
HeLa genomic DNA
H2O -ve control
Control subjects
1 2 3 4 5 6 7 8 9 10
HeLa genomic DNA
H2O -ve control
614 bp

detection of the amplified PCR product in PBMC RNA samples was not due to the genomic DNA contamination alone.

4.2.3. Sequence analysis of IDDMK$_{1,2}$22 specific PCR product

In the different sets of experiments described above using different primers - the U3/R-poly(A) primer set and the 5'SAg/3'SAg primer set - our results show consistently that there is no difference in the expression of the endogenous retrovirus IDDMK$_{1,2}$22 between IDDM patients and non-diabetic control subjects. To determine whether the uniformly amplified PCR product did indeed come from IDDMK$_{1,2}$22, we sequenced the amplified PCR product using the U3 primer. We did not find a sequence identical to that of IDDMK$_{1,2}$22. We did, however, find similar sequences, sharing 90-93% sequence homology with that of IDDMK$_{1,2}$22 (Figure 10).

4.2.4. Discussion

This study showed that an endogenous retrovirus, related to the HERV-K family, was certainly present equally

Figure 10. Sequence of U3/R-poly(A) amplified PCR product. Row 1 is the published sequence of IDDMK$_{1,2}$22 (Genbank Accession No. AF012335). Rows 2-6 are sequences obtained from IDDM patients (DM = diabetes mellitus). Rows 7-10 are sequences obtained from normal control subjects (NC = normal control). 90 to 93% homology was observed between our sequences and that of IDDMK$_{1,2}$22. Only the non-matching nucleotides are shown.

IDDMK$_{1,2}$22	AGGTATTGTC	CAAGGTTTCT	CCCCATGTGA	TAGTCTGAAA	TATGGCCTCG	TGGGAAGGGA	AAGACCTGAC
DM 9	----------	----------	----------	-- -------	----------	----------	----------
DM 10	----------	----------	----------	----------	----------	----------	----------
DM 11	----------	----------	----------	----------	----------	----------	----------
DM 12	----------	----------	----------	----------	----------	----------	----------
DM 13	----------	----------	----------	----------	----------	----------	----------
NC 18	----------	----------	----------	----------	----------	----------	----------
NC 21	----------	----------	----------	----------	----------	----------	----------
NC 22	----------	----------	----------	----------	----------	----------	----------
NC 23	----------	----------	----------	----------	----------	----------	----------

IDDMK$_{1,2}$22	CATCCCCCAG	ACCAACACCC	GTAAAGGGTC	TGTGCTGAGG	AGGATTAGTA	TAAGAGGAAA	GCATGCCTCT
DM 9	-G--------	C--G------	----------	----------	----------	A--------G	-C--C-----
DM 10	-G--------	C--G------	----------	----------	----------	A--------G	-A--------
DM 11	-G--------	C--G------	----------	----------	----------	A--------G	-A--------
DM 12	-G--------	C--G------	----------	----------	----------	A--------G	-A--C-----
DM 13	-G--------	C--G------	----------	----------	----------	A--------G	-A--------
NC 18	-G--------	C--G------	----------	----------	----------	A--------G	----C-----
NC 21	-G--------	C--G------	----------	----------	----------	A--------G	----C-A---
NC 22	-G--------	C--G------	----------	----------	----------	A--------G	-A--C-----
NC.23	-G--------	C--G------	----------	----------	----------	A--------G	----C-----

IDDMK$_{1,2}$22	TGCAGTTGAG	AGAAGAGGAA	GACATCTGTC	TCCTGCCCAT	CCCCTGGGCA	ATGGAATGTC	TCAGTATAAA
DM 9	-------T--	-C------G-	AG---TC---	--------G-	--- ------	--T-------	--G-------
DM 10	-------T-A	-C-------	AG ---C---	--------G-	----------	----------	--G-------
DM 11	-------T-A	-C----A-C-	AG----C---	--------G-	T-- ------	----------	C-G-------
DM 12	----A--T-A	-C----A-G-	AG---TC---	--------G-	T-- ------	--T-------	--G-------
DM 13	-------T-A	-C----A-G-	AG----C---	--------G-	T-- ------	----------	--G-------
NC 18	-T-----T-A	-C----A-G-	AG---TC---	--------G-	T---------	--T-------	--G-------
NC 21	----A--T-A	-C------G-	AG---TC---	--------G-	T-- ------	--T-------	--G-------
NC 22	----A----A	-C----A---	AG----C---	--------G-	T-- ------	----------	C-G-------
NC.23	----A--T-A	-C--------	AG----C---	-----G--G-	--- ------	----G-----	C-G-------

IDDMK$_{1,2}$22	ACCCGATTGA	ACATTCCATC	TACTGAGATA	TGTATATCTA	AAAGCACAGC	ACTTGATCCT	TTACCTTGTC
DM 9	---------T	TTGC------	----------	--C-------	----------	----A-----	----------
DM 10	---------T	TTGC------	----------	--C-------	----------	----A-----	----A-----
DM 11	---------T	TTGC------	C-----A---	--C-------	----------	----A-----	----------
DM 12	---------T	TTGC------	----------	--C-------	----------	----A-----	----A-----
DM 13	---------T	TTGC------	------A---	--C-------	----------	----A-----	----A-----
NC 18	----T---TT	TTCC----A-	------A---	--CC------	--------T-	----A-----	----------
NC 21	---------T	TTGC------	------A---	A-C-------	----------	----A-----	----A-----
NC 22	---------T	TTGC----A-	--A---A---	A-C-------	----------	----A-----	----------
NC.23	----C----T	TTGC------	--A---A---	A-C-------	----------	----A-----	----------

IDDMK$_{1,2}$22	TATGATGCAA	ACACCTTTGT	TCACGTGTTT	GTCTGCTGAC	CCTCTCCCCA	CTATTGTCTT	GTGACCCTGA
DM 9	----------	-G--------	----------	----------	----------	-A--------	----------
DM 10	----------	-G--------	----------	----------	----------	-A--------	----------
DM 11	----------	-G--------	----------	----------	----------	-A--------	----------
DM 12	----------	-G--------	----------	----------	----------	-A--------	----------
DM 13	----------	-G--------	----------	----------	----------	-A--------	----------
NC 18	----------	-G--------	----------	----------	----------	-A--------	----------
NC 21	----------	-G--------	----------	----------	----------	-A--------	----------
NC 22	----------	-A--------	----T-----	----------	----------	-A--------	----------
NC.23	----------	-A--------	----T-----	----------	----------	-A--------	----------

IDDMK$_{1,2}$22	CACATCTCCC	TCA GGAGAA	ACACCCACGA	ATGATCAATA	AATACTAAGG	GGACTCAAAA	A
DM 9	------C---	--TTC-----	--------A-	----------	G---------	----------	-
DM 10	------C---	--T T-----	--------A-	----------	----------	----------	-
DM 11	------C---	--TTC-----	--------A-	----------	----------	----------	-
DM 12	------C---	--TTT-----	--------A-	----------	----------	----------	-
DM 13	------C---	--TTC-----	--------A-	----------	----------	----------	-
NC 18	------C---	--T ------	--------A-	----------	----------	----------	-
NC 21	------C---	--TTC-----	--------A-	----------	----------	----------	-
NC 22	------C---	--TTC-----	--------A-	----------	----------	----------	-
NC.23	------C---	TCT TC----	--------A-	----------	----------	----------	-

in IDDM patients and non-diabetic control subjects. Our finding is quite contradictory to the report by Conrad et al. (Conrad et al., 1997). A possible explanation for the distinct difference in the identification of $IDDMK_{1,2}22$ between the IDDM patients and the control subjects found by Conrad et al. could be two separate sets of experiments. In Conrad's report the schedule of blood collection and RNA preparation showed that all the IDDM patients were investigated together on one occasion, while the control subjects were investigated together on another occasion. In such circumstances, an error in one set of experiments might account for the results found by Conrad et al. It is also conceivable that there was a significant genetic variability between the subject pools in the two different studies and/or significant differences in the specific exposure to other infectious agents, including certain viruses and bacteria. These diverse infectious agents possibly activate endogenous retroviral genes differently, which could be reflected in the differences in the detection of a retroviral gene in the samples collected from the disparate locations.

In conclusion, a sequence identical to that of $IDDMK_{1,2}22$ was not detected in either the plasma or

peripheral blood lymphocytes from IDDM patients or non-diabetic control subjects. A related human endogenous retroviral gene with 90±93% sequence homology with IDDMK 1,2 22, however, was equally present in both diabetic and non-diabetic subjects. Therefore, we conclude that an endogenous retroviral gene with high sequence homology with $IDDMK_{1,2}22$ is not specific for IDDM patients but is ubiquitous, indicating that this retrovirus is unlikely to be associated with the development of autoimmune diabetes in humans.

4.3. RT mediated persistence of EMC-D virus and its role in the pathogenesis of murine IDDM

4.3.1. Incidence of diabetes in EMC-D infected DBA/2 mice.

EMC-D virus produces diabetes in genetically susceptible strains of mice including DBA/2 (Yoon et al., 1980). This mouse strain has been used in the recent past to examine the role of macrophages in the pathogenesis of diabetes after low dose infection with EMC-D virus (Hirasawa et al., 1999; Hirasawa et al., 1997; Hirasawa et

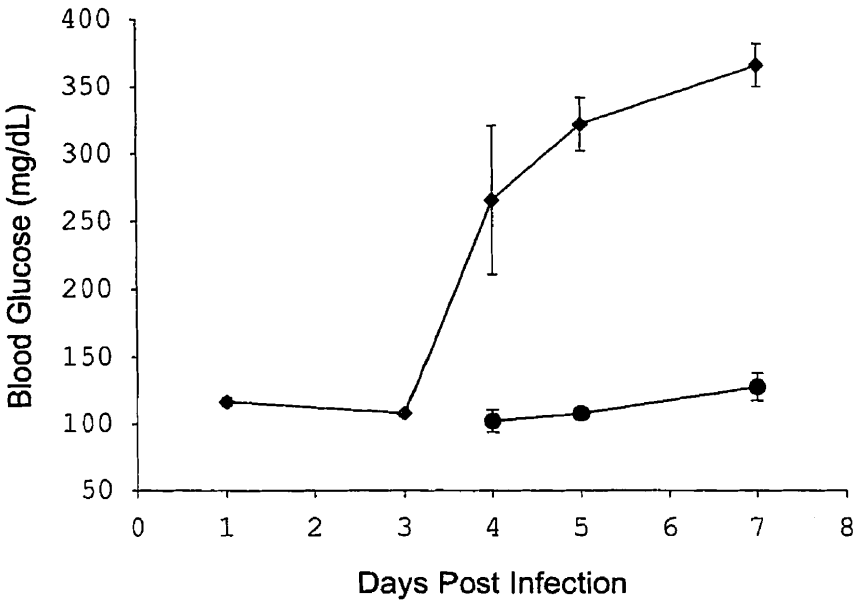
al., 1996). Diabetes in this low dose model is not produced by direct cytolytic destruction of pancreatic β-cells but indirectly by specific immune activity in response to viral infection. It was for this reason that this model was chosen for our studies on reverse transcriptase mediated persistence of EMC-D virus induced IDDM.

Eight to 12 week old male DBA/2 mice were injected with 100pfu of EMC-D virus. Diabetes developed within 5 days p.i.. The cumulative incidence of diabetes in virus infected mice was 69% at 5 days p.i. while none of the placebo infected mice became diabetic (Figure 11).

4.3.2. Detection of reverse transcribed EMC-D cDNA in the pancreata of EMC-D virus infected animals

Evidence of a cDNA form of the EMC-D viral RNA genome was found in the pancreata of DBA/2 mice infected with a low dose (100pfu) of EMC-D virus. At this dosage EMC-D does not cause direct cytolytic destruction of pancreatic β-cells but is instead involved in the recruitment of immune cells and the initiation of an autoimmune response. We initially examined the appearance of EMC-D cDNA at 1, 3, and 5 weeks p.i. and found that 7/9, 5/9, and 4/9 animals exhibited

Figure 11. Blood glucose levels in EMC-D infected vs. control mice. Male DBA/2 mice aged 8 weeks (n=36) were infected with 100pfu of EMC-D virus (♦) or the appropriate dilution of L929 cell lysate (•).


400
350
300
250
200
150
100
50
Blood Glucose (mg/dL)
0
1
2
3
4
5
6
7
8
Days Post Infection

cDNA at the respective time points (Figure 12). Further examination at time points within the 1st week p.i. revealed that consistent detection of EMC-D cDNA was not seen until the 4th day p.i.(Figure 13) which coincides with the first signs of elevated blood glucose (Figure 11). EMC-D cDNA could not be detected in individual pancreatic genomic DNA isolates of either DBA/2 mice infected with an L929 cell lysate or kidney tissue from DBA/2 mice infected with 100pfu EMC-D (data not shown)

4.3.3. Confirmation of assay fidelity by examination of procedural artefacts

PCR is a powerful tool for the detection of rare sequences but the very ability to amplify trace amounts of genetic information creates a significant bias. The incidental amplification of non-native template sequences can lead to the detection of false positives. If the purpose of a study is the detection of sequences whose origin is attributed to reverse transcription by endogenous factors, such false positive results can be a distressing phenomenon (Weiss and Kellam, 1997). For this reason our

Figure 12. Generation of EMC-D cDNA from pancreatic genomic DNA by EMC-D VP1 primer specific PCR. Genomic DNA from the pancreata of male DBA/2 mice was isolated at 1 week (A), 3 weeks (B), and 5 weeks (C) after infection with 100pfu EMC-D virus and subjected to PCR amplification with primers specific for the VP1 gene of EMC-D virus. Lanes 1-9 refer to individual mice.

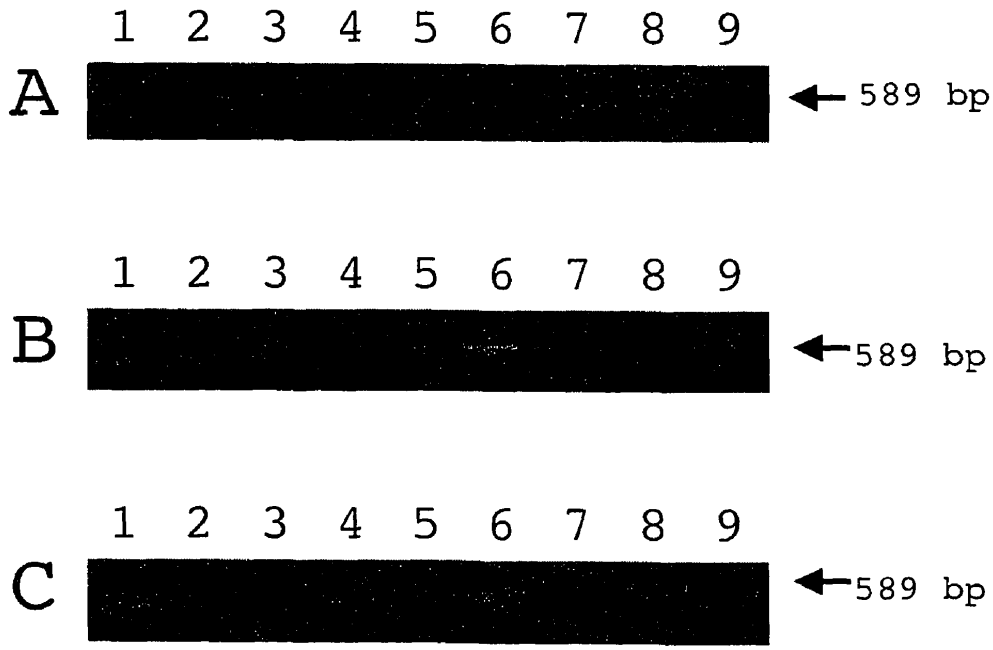
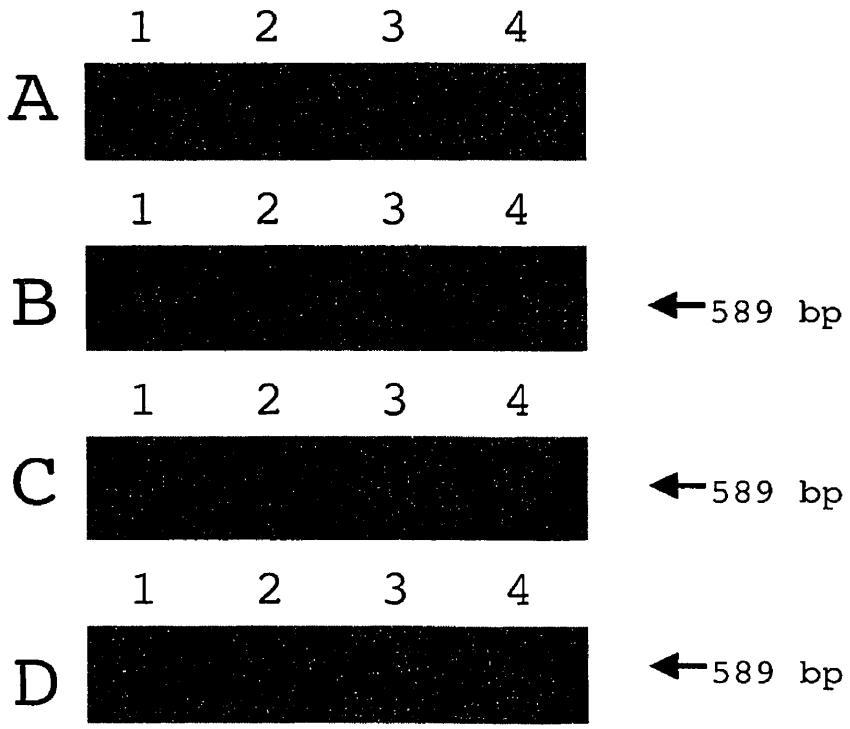

1 2 3 4 5 6 7 8 9
A
589 bp
1 2 3 4 5 6 7 8 9
B
589 bp
1 2 3 4 5 6 7 8 9
C
589 bp

Figure 13. Generation of EMC-D cDNA from pancreatic genomic DNA by EMC-D VP1 primer specific PCR. Genomic DNA from the pancreata of male DBA/2 mice was isolated at 1 day (A), 3 days (B), 4 days (C), and 5 days (D) after infection with 100pfu EMC-D virus and subjected to PCR amplification with primers specific for the VP1 gene of EMC-D virus. Lanes 1-9 refer to individual mice.


1 2 3 4
A
1 2 3 4
B
589 bp
1 2 3 4
C
589 bp
1 2 3 4
D
589 bp

standard assay for detection of reverse transcribed EMC-D viral genome was systematically tested for bias.

Certain Taq polymerases are known to possess an intrinsic reverse transcriptase activity (Chang et al., 1997; Lugert et al., 1996; Maudru and Peden, 1997). To confirm whether our Taq polymerase exhibited such activity a PCR reaction mixture was prepared as in section 4.3.2. and run according to a modified cycle program. The standard assay protocol was altered to include a RT permissive incubation step of 37°C for 60min prior to the usual PCR cycling program. We were able to detect, by a single round of PCR using EMC-D VP1 primers, an artefact from a RNA template mixture containing as little as 0.1ug of EMC-D viral RNA (Figure 14A). Our detection sensitivity was increased by 10 fold when the PCR product was transferred to nylon membrane and subjected to southern blotting with an EMC-D VP1 specific probe (Figure 14B). When the same assay was carried out under standard, non-RT permissive, conditions we were not able to amplify EMC-D VP1 specific PCR products even from as much as 1ug EMC-D viral RNA (Figure 16A). Southern blotting with EMC-D VP1 specific probe as above did not improve our detection sensitivity (Figure 15B).

Figure 14. RT permissive PCR of EMC-D viral RNA. EMC-D viral RNA was amplified under conditions permissive for the generation of reverse transcriptase product by Taq polymerase. (A) PCR. (B) Southern blot of PCR products in A. Lane 1: 1ug EMC-D viral RNA template. Lane 2: 0.1ug EMC-D viral RNA template. Lane 3: 0.01ug EMC-D viral RNA template. Lane 4: 0.001ug EMC-D viral RNA template.

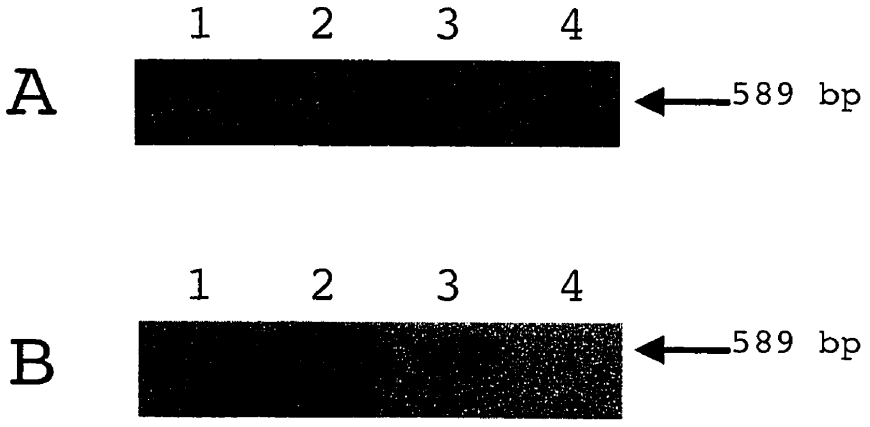
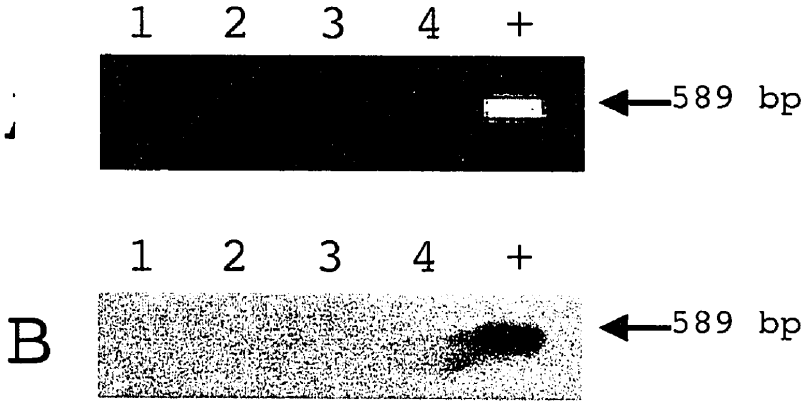

1
2
3
4
A
589 bp
1
2
3
4
B
589 bp

Figure 15. Standard, non-RT permissive PCR of EMC-D viral RNA. EMC-D viral RNA was amplified under standard conditions, non-permissive for the generation of reverse transcriptase product by Taq polymerase. (A) PCR. (B) Southern blot of PCR products in A. Lane 1: 1ug EMC-D viral RNA template. Lane 2: 0.1ug EMC-D viral RNA template. Lane 3: 0.01ug EMC-D viral RNA template. Lane 4: 0.001ug EMC-D viral RNA template. +: positive control, $1:10^5$ dilution of EMC-D reverse transcriptase product (prepared with commercial RT, Supercript II).


1 2 3 4 +
589 bp
B
1 2 3 4 +
589 bp

The ability of Taq polymerase to reverse transcribe and amplify EMC-D viral RNA in a PCR reaction mixture containing genomic DNA was also tested. Varying amounts of EMC-D viral RNA was mixed with lug of pancreatic genomic DNA from DBA/2 mice for use as template in RT permissive PCR as above. In contrast to the use of viral RNA alone as template the addition of genomic DNA to our PCR reaction mixture resulted in the loss of the ability of Taq polymerase to reverse transcribe and amplify an EMC-D VP1 specific sequence (Figure 16).

The possibility existed that the viral RNA within virions vs. free cytoplasmic viral RNA could survive our isolation procedures for genomic DNA intact. We therefore mixed live virus with pancreatic tissue from uninfected DBA/2 mice and isolated genomic DNA according to our standard protocol. RT permissive PCR was carried out on lug of isolated genomic DNA but we were unable to detect an EMC-D VP1 specific PCR product directly (Figure 17A) or by southern blotting with EMC-D VP1 specific probe (Figure 17B).

Figure 16. RT permissive PCR of EMC-D viral RNA in the presence of genomic DNA. 0.1ug of EMC-D viral RNA was added to 1ug of genomic DNA isolated from uninfected male DBA/2 mice (n=8, Lanes 1-8) and amplified under conditions permissive for the generation of RT product by Taq polymerase. (A) PCR. (B) Southern blot of PCR products in A. +: positive control, $1:10^5$ dilution of EMC-D reverse transcriptase product (prepared with commercial RT, Supercript II).

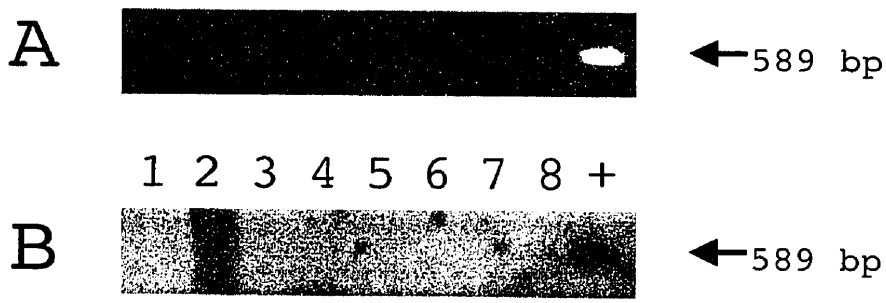
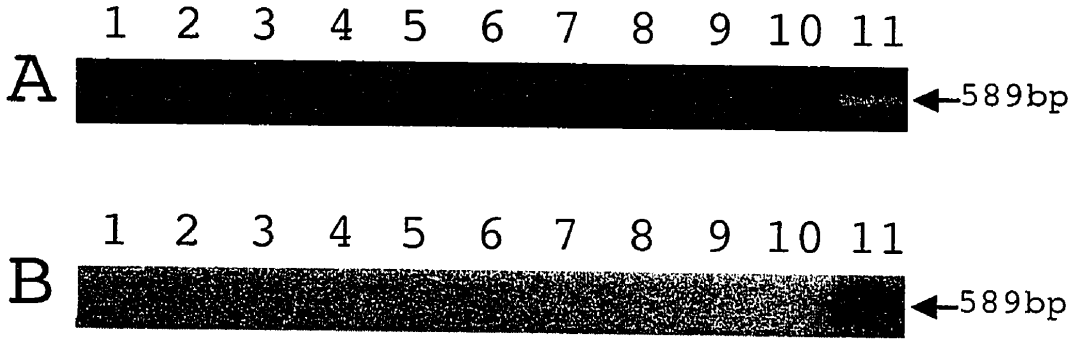

A
589 bp
1 2 3 4 5 6 7 8 +
B
589 bp

Figure 17. RT permissive PCR of live EMC-D virus co-isolated with pancreatic genomic DNA. 10^7 pfu of live EMC-D virus was mixed with a ½ frozen pancreas from male DBA/2 mice (n=10, Lanes 1-10) and genomic DNA was immediately isolated. 1ug of genomic DNA was subsequently amplified under conditions permissive for the generation of RT product by Taq polymerase. (A) PCR. (B) Southern blot of PCR products in A. Lane 11: positive control, $1:10^5$ dilution of EMC-D reverse transcriptase product (prepared with commercial RT, Supercript II).


1 2 3 4 5 6 7 8 9 10 11
A
589bp
1 2 3 4 5 6 7 8 9 10 11
B
589bp

4.3.4. Removal of PCR amplified EMC-D viral RNA signal by RNase A treatment

The efficacy of RNase A treatment in the removal of the PCR amplified EMC-D viral RNA signal observed in section 4.3.3. was examined. The same EMC-D viral RNA used as template for the PCR shown in Figure 14 was also treated with RNase A at a final concentration of 2mg/ml. The addition of RNase A at this concentration was able to prevent the formation of the artefactual PCR product shown in Figure 14 as evidenced by direct PCR (Figure 18A) and southern blotting using an EMC-D VP1 specific probe (Figure 18B). It should be noted that though this concentration of RNase A was used as part of our standard operating procedure for the isolation of genomic DNA, the minimum concentration of the enzyme required to prevent the generation of a PCR artefact was 200pg/ml (Figure 19A). Thus, we are routinely using a concentration of enzyme that is $1x10^7$ fold greater than necessary.

To address concerns that the addition of a high concentration of RNase A could affect the normal efficiency of our PCR reaction we titrated the inhibitory effect of RNase A using EMC-D RT product (prepared with the

Figure 18. RNase A treatment of EMC-D viral RNA prior to RT permissive PCR. EMC-D viral RNA treated with RNase A was amplified under conditions permissive for the generation of reverse transcriptase product by Taq polymerase. (A) PCR. (B) Southern blot of PCR products in A. Lane 1: 1ug EMC-D viral RNA template. Lane 2: 0.1ug EMC-D viral RNA template. Lane 3: 0.01ug EMC-D viral RNA template. Lane 4: 0.001ug EMC-D viral RNA template. Lane 5: 1ug EMC-D viral RNA template. Lane 6: 0.1ug EMC-D viral RNA template. Lane 7: 0.01ug EMC-D viral RNA template. Lane 8: 0.001ug EMC-D viral RNA template. Lanes 1-4 were untreated. RNase to a final concentration of 2 mg/ml was added to Lanes 5-8.

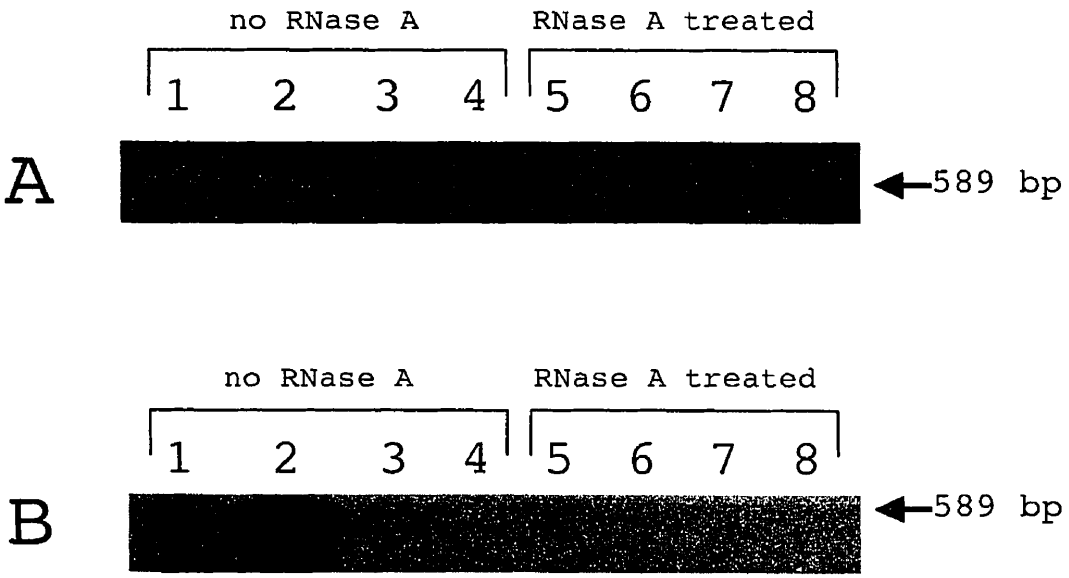
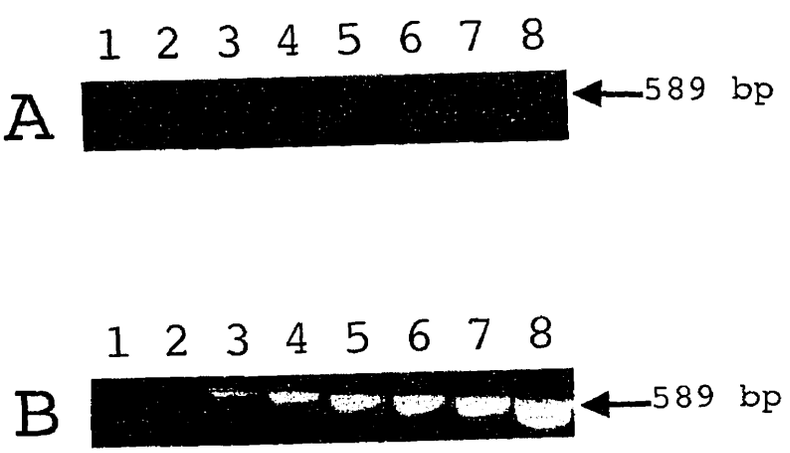

no RNase A
RNase A treated
1 2 3 4 5 6 7 8
A
589 bp
no RNase A
RNase A treated
1 2 3 4 5 6 7 8
B
589 bp

Figure 19. Titration of the inhibitory activity of RNase A. (A) 0.1ug EMC-D viral RNA template amplified under conditions permissive for the generation of reverse transcriptase product by Taq polymerase. (B) $1:10^5$ EMC-D RT product template (5ul) amplified under standard conditions, non-permissive for the generation of reverse transcriptase product by Taq polymerase. Lane 1: final concentration RNase A = 2mg/ml. Lane 2: 200ug/ml. Lane 3: 20ug/ml. Lane 4: 2ug/ml. Lane 5: 200ng/ml. Lane 6: 20ng/ml. Lane 7: 2ng/ml. Lane 8: 200pg/ml.

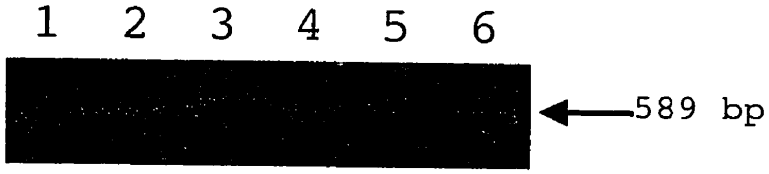

1 2 3 4 5 6 7 8
A
589 bp
1 2 3 4 5 6 7 8
B
589 bp

commercial RT, Superscript II). At a final concentration of 200ug/ml in the PCR reaction mixture RNase A was capable of inhibiting our standard PCR (Figure 19B). This was not, however, the likely concentration of RNase A in the genomic DNA eluate after elution. In lieu of an assay for RNase A activity in our genomic DNA eluate, we carried out our standard genomic DNA isolation protocol on a mixture containing all the appropriate buffers with RNase A at a concentration of 2mg/ml but lacking any tissue or other source of genomic DNA. The eluate from this isolation was serially diluted and titrated against EMC-D RT product as above. It was found that this eluate did not inhibit PCR at any concentration (Figure 20).

4.3.5. Sequence confirmation of EMC-D VP1 primer specific PCR product

To ensure that the PCR products generated in section 4.3.2. were not the result of non-specific amplification we conducted sequence analysis on two of our sample PCR products. The PCR products visualized in Lanes 1 and 2 of Figure 12A were purified separately and sequenced along with the positive control product. Analysis of these two

Figure 20. Titration of the inhibitory activity of eluted RNase A. $1:10^5$ EMC-D RT product template (5ul) amplified under standard conditions, non-permissive for the generation of reverse trancriptase product by Taq polymerase. Lane 1: undiluted eluate (5ul). Lane 2: 1:10. Lane 3: 1:100. Lane 4: 1:1000. Lane 5: $1:10^4$. Lane 6: $1:10^5$.


1 2 3 4 5 6
589 bp

sequences and the EMC-D RT product routinely used as positive control revealed 98.8 to 99.7% homology with the published sequence of EMC-D (GenBank accession no. M22458 J04355) (Figure 21).

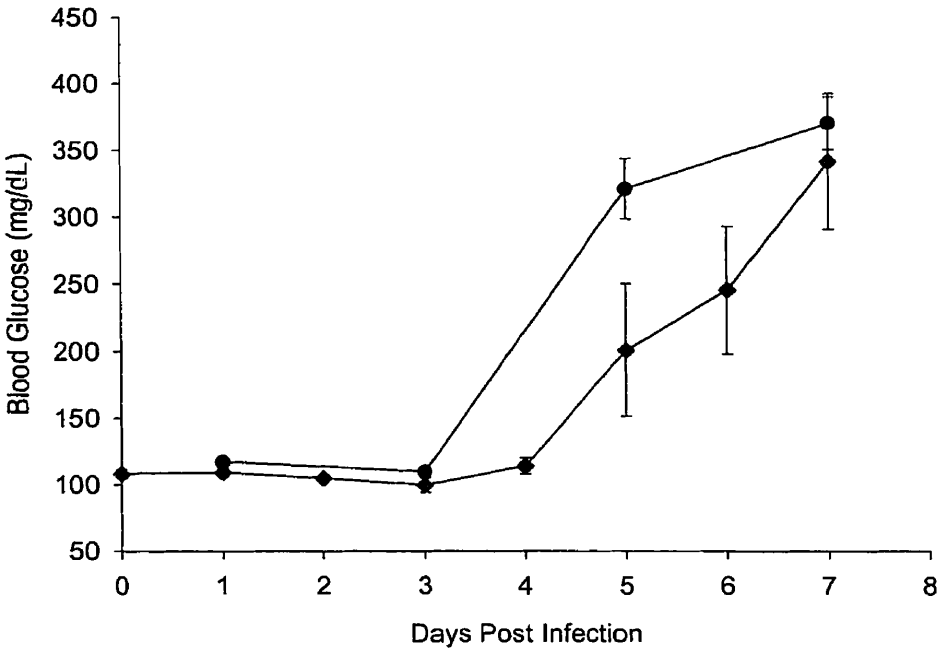
4.3.6. The effect of AZT treatment on the persistence of EMC-D virus and development of diabetes

We tested the effect of the antiretroviral agent AZT, which inhibits the activity of reverse transcriptase on the incidence and characteristics of diabetes development in EMC-D virus infected DBA/2 mice. Mice in both treatment groups exhibited normal blood glucose levels during the first 3 days p.i.. At 5 days p.i. the incidence of diabetes was 25% in AZT treated mice vs. 69% in untreated mice. The average blood glucose level at 5 days p.i. was 200.5 mg/dl in AZT treated mice vs. 320.9 mg/dl in untreated mice ($p<0.02$, students t-test). At 7 days p.i. the incidence of diabetes increased to 63% in AZT treated mice and 89% in untreated mice (Figure 22) with similar average blood glucose levels; 341.5 mg/dl and 370.2 mg/dl in treated and untreated mice respectively. Pancreata were harvested at 7 days p.i. and assayed for the cDNA form of EMC-D. The cDNA

Figure 21. Sequence analysis of PCR products. PCR products from standard EMC-D VP1 primer specific amplification generated from pancreatic genomic DNA samples 1 (PW1-1) and 2 (PW1-2) harvested 1 week p.i.. (EMC-D): published sequence of the amplified region (GenBank accession no. M22458 J04355). (RT): sequence of the PCR amplified EMC-D RT product control.

```
EMC-D  2925 ACAGTTTGAC CCCGCTTATG ACCAGCTGAG ACCCGAGCGC CTAACGGAGA TTTGGGGCAA
PW1-1       ---------- ---------- ---------- ---------- ---------- ----------
PW1-2       ---------- ---------- ---T------ C--------- ---------- ----------
RT          ---------- ---------- ---C-C---- ---------- ---------- ----------

EMC-D  2985 TGGAAATGAA GAAACTTCAA AAGTCTTTCC ATTAAAATCA AAACAAGACT ATTCTTTCTG
PW1-1       ---------- ---------- ---------- ---------- ---------- ----------
PW1-2       ---------- ---------- ---------- ---------- ---------- ----------
RT          ---------- ---------- ---------- ---------- ---------- ----------

EMC-D  3045 TCTGTTTTCC CCTTTTGTTT ATTATAAATG TGATCTAGAG GTTACTCTGA GTCCTCACAC
PW1-1       ---------- ---------- ---------- ---------- ---------- ----------
PW1-2       ---------- ---------- ---------- ---------- ---------- ----------
RT          ---------- ---------- ---------- ---------- ---------- ----------

EMC-D  3105 CTCTGGCAAT CATGGGCTGT TGGTTAGGTG CTGCCCAACT GGAACGCCAG CCAAGCCAAC
PW1-1       ---------- ---------- ---------- G--------- ---------- ----------
PW1-2       ---------- ---------- ---------- G--------- ---------- ----------
RT          ---------- ---------- ---------- G--------- ---------- ----------

EMC-D  3165 CACTCAAGTA CTGCATGAAG TGAGCTCGCT CTCGGAAGGT AGAACCCCTC AGGTTTACAG
PW1-1       ---------- ---------- ---------- ---------- ---------- ----------
PW1-2       ---------- ---------- ---------- ---------- ---------- ----------
RT          ---------- ---------- ---------- ---------- ---------- ----------

EMC-D  3225 TGCTGGGCCA GGGATCTCGA ATCAAATCTC TTTCGTGGTG CCGTACAATT CTCCCCTTTC
PW1-1       ---------- ---------- ---------- ---------- ---------- ----------
PW1-2       ---------- ---------- ---------- ---------- ---------- ----------
RT          ---------- ---------- ---------- ---------- ---------- ----------

EMC-D  3285 AGTCCTGCCA GCCGTGTGGT ATAATGGTCA CAAGCGGTTT GACAACACTG GAAGTCTGGG
PW1-1       ---------- ---------- ---------- ---------- ---------- ----------
PW1-2       ---------- ---------- ---------- ---------- ---------- ----------
RT          ---------- ---------- ---------- ---------- ---------- ----------

EMC-D  3345 AATAGCTCCC AACTCGGATT TTGGAACTTT GTTCTTTGCG GGCACAAAAC CAGACATAAA
PW1-1       ---------- ---------- ---------- ---------- ---------- ----------
PW1-2       ---------- ---------- ---------- ---------- ---------- ----------
RT          ---------- ---------- ---------- ---------- ---------- ----------

EMC-D  3405 GTTTACAGTG TACCTAAGGT ACAAAAATAT GAGAGTTTTT TGCCCGCGTC CAACGGTGTT
PW1-1       ---------- ---------- ---------- ---------- ---------- ----------
PW1-2       ---------- ---------- ---------- ---------- ---------- ----------
RT          ---------- ---------- ---------- ---------- ---------- ----------

EMC-D  3465 CTTCCCATGG CCCTCCTCAG GAGATAAGAT AGACATGACC CCGAGAGCT- ----------
PW1-1       ---------- ---------- ---------- ---------- ----G----- ----------
PW1-2       ---------- ---------- ---------- ---------- ----A----- ----------
RT          ---------- ---------- ---------- ---------- ---GAGC--- ----------
```

Figure 22. Effect of AZT on blood glucose levels. Male DBA/2 mice aged 12 weeks (n=8) were infected with 100pfu EMC-D virus and either pre- and continuously treated with an oral dose of 2.5 mg/ml AZT (♦) or untreated (•).

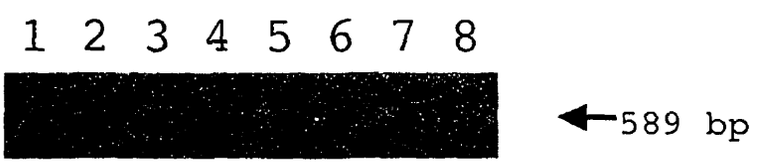

450
400
350
300
250
200
150
100
50
Blood Glucose (mg/dL)
0
1
2
3
4
5
6
7
8
Days Post Infection

form of EMC-D was detected in every pancreas isolated (Figure 23).

4.3.7. Discussion

The replication cycle of EMC-D virus does not require a cDNA intermediate. There are at present two well known virus families that encode the enzyme reverse transcriptase, the retroviridae and the hepadnaviridae (Hepatitis B virus). Reverse transcriptase is an RNA dependent DNA polymerase that converts the RNA genome of the former and an RNA intermediate of the latter into DNA. Evidence from two independent reports indicates that reverse transcription of a variety of RNA viruses is possible through the action of reverse transcriptase encoded by endogenous retroviruses (Klenerman et al., 1997; Zhdanov, 1975). The vast majority of viruses implicated in the pathogenesis of IDDM are RNA viruses (Table 4) and the prospect of a mechanism, i.e. reverse transcription, capable of linking the etiology of these viruses to IDDM along a common pathway is intriguing. Persistence of RNA viruses as a unifying theme in the pathogenesis of IDDM would have a potentially tremendous impact on our

Figure 23. Detection of EMC-D cDNA in the pancreata of AZT treated mice. Male DBA/2 mice aged 12 weeks (n=8, Lanes 1-8) were infected with 100pfu EMC-D virus. Standard PCR was conducted on 1ug of pancreatic gDNA, isolated at 1 week p.i..


1 2 3 4 5 6 7 8
589 bp

perspective of the events involved in the initiation of the autoimmune process leading to pancreatic β-cell destruction.

We found evidence that the diabetogenic RNA virus EMC-D could be reverse transcribed in the pancreata of infected DBA/2 mice. The earliest consistent detection of the cDNA form by PCR was at 4 days p.i. with maximal expression evident at 1 week p.i.. The incidence of cDNA declined at later time points (Figures 12 and 13). This decline is likely due to the destruction and clearance of the pancreatic β-cells harboring EMC-D as the pervasiveness of islet destruction and resulting severity of diabetes progresses. The individual blood glucose levels of mice from Figure 13 exhibiting the cDNA form of EMC-D virus were elevated. Those mice which were negative for the cDNA form of the virus conversely exhibited normal blood glucose levels (data not shown). Thus there was a perfect relationship between expression of EMC-D cDNA and the incidence of elevated blood glucose at 1, 3, 4, and 5 days p.i.. The individual blood glucose levels of mice from Figure 12 exhibiting the cDNA form of EMC-D virus were elevated at 1 week p.i. with two exceptions. The correspondence between elevated blood glucose and EMC-D cDNA did not hold at 3 and 5 weeks p.i. (data not shown).

In the case of the two mice that exhibit EMC-D cDNA at 1 week p.i. but do not demonstrate elevated blood glucose levels the disease process may be ongoing. These animals might have developed diabetes at a later date but it was necessary to euthanize them in order to carry out our assay for the cDNA form of EMC-D. The possibility of harvesting pancreatic biopsy samples from mice at intervals p.i. was briefly considered but it was immediately decided that such an approach would introduce significant bias and, more significantly, was logistically unfeasible. The possibility also exists that with the potential involvement of the immune system in the destruction of EMC-D virus infected β-cells a component necessary for said destruction is not always activated in response to the appropriate viral insult in every infected animal. The occurrence of clinical diabetes in mice which do not exhibit EMC-D cDNA at 3 and 5 weeks post infection is most likely due to extensive destruction of the β-cells harboring the cDNA form, precluding detection.

Viral sequences lack introns and as a result a definitive assay capable of distinguishing the viral RNA genome from reverse transcribed cDNA is not available. Extreme care must therefore be taken the preparation of

experimental PCR templates and reactions. Multiple control experiments were thus conducted to determine the fidelity and sensitivity of our PCR based assay. It was found that under experimentally altered, i.e. permissive, conditions the generation of a positive PCR signal artefact from a viral RNA template was possible (Figure 14). The reverse transcriptase activity of Taq polymerases and other DNA polymerases has been documented so this was not a surprising result (Chang et al., 1997; Lugert et al., 1996; Maudru and Peden, 1997). This artefact could not be reproduce under normal assay conditions (Figure 15).

We also examined the production of this artefact in the presence of genomic DNA and found that even with RT permissive RT conditions the production of a PCR artefact from viral RNA was not possible. This was not an expected result but advantageous, and a favourable condition for our standard assay. The likely explanation for this phenomenon is that the genomic DNA present in the PCR reaction mixture acts to 'quench' formation of the spurious PCR product by 'soaking up' the DNA PCR primer. This hypothesis is based on two principles: 1) the preferential affinity of DNA for DNA vs. DNA for RNA which is, of course counterbalanced by the homology based specificity of the potential DNA

primer/RNA template interaction and 2) the preference of DNA dependent polymerases such as Taq for their natural DNA substrate. 'DNA quenching' is, in fact, a recognized method for the suppression of false positives in PCR applications (Lugert et al., 1996).

It is possible that viral RNA could find its way into our final genomic DNA isolate by virtue of its protected state within intact virions. Our DNA isolation procedure is designed to exclude free cytoplasmic RNA but we were uncertain whether exclusion of intact virions would be complete. It was demonstrated that a PCR artefact could not be generated under RT permissive conditions from genomic DNA isolated from a live virus/tissue mixture (Figure 16). The addition of the commercial reverse transcriptase, Superscript II, to the genomic DNA isolate and subsequent PCR did not result in the generation of a PCR artefact (data not shown).

Experimental examination of the efficacy of RNase A treatment in the removal of PCR artefacts generated from pure viral RNA templates revealed that as little as 200 pg/ml of the enzyme was sufficient to completely prevent incidental positive signals (Figure 19). As we employ a final concentration of the enzyme that is $1x10^7$ fold greater

our confidence in the ability of our assay to exclude RNA is understandable.

To confirm that the amplified product was indeed EMC-D VP1 specific we sequenced two of our positive PCR products. It was revealed that greater than 98.8% homology existed with the published sequence of EMC-D.

The effect of reverse transcription on the persistence of EMC-D and the potentially related effect on development of diabetes was examined by the inhibition of reverse transcriptase activity. The reverse transcriptase specific antiretroviral agent AZT was administered orally in the drinking water of EMC-D virus infected animals. A significant difference in the average blood glucose levels of infected/AZT treated vs. infected/untreated was observed at 5 days p.i. but this difference was no longer significant by 7 days p.i.. The kinetics of diabetes development was thus altered by a maximum of 2 days but administration of AZT did not prevent onset of the disease (Figure 22). When pancreata were harvested at 7 days p.i. it was revealed that treatment of mice with high dose AZT orally was not effective in preventing the formation of EMC-D cDNA at this time point (Figure 23).

Though the cDNA formation phenomenon appears to be reliable the relevance of the cDNA form of EMC-D to IDDM pathogenesis must still be demonstrated definitively. The finding that AZT was capable of delaying the onset of virus induced IDDM is an encouraging result. The subsequent determination that the delay was achieved despite incomplete inhibition of cDNA formation is not damaging to our hypothesis but neither does it prove that endogenous retrovirus induced reverse transcription of EMC-D is necessary for development of IDDM in infected DBA/2 mice.

4.4. RT mediated persistence of CB4 virus and its role in the pathogenesis of human IDDM

4.4.1. Case Report: A recently deceased IDDM patient

On January 17th, 2000 a female patient, aged 10 years, was admitted to hospital in Cranbrook, British Columbia with general malaise and persistent flu-like symptoms. She was transferred to the Alberta Children's Hospital (ACH) in Calgary, Alberta on January 19th as her condition worsened and she was diagnosed with acute diabetic ketoacidosis at that time. A chest x-ray revealed a pervasive congestion of

her lungs and it was indicated that her white blood cell count had dropped from 25000 to 2 during her hospitalization, consistent with a severe infection. The patient passed away on January 19th; cause of death listed as acute cerebral edema. The earliest indication of illness in this child was the parents' report of polyuria dating from Christmas 1999.

Anecdotal reports indicate that at least 5 diabetes related deaths have occurred at the ACH during the past 15 years. Three deaths were due to shock. Two deaths were in Cranbrook; 1 each in Red Deer, Lethbridge, and Medicine Hat. The death of this child brings the number deaths occurring in individuals from Cranbrook to 3.

Pancreatic tissue was obtained subsequent to organ harvest for transplantation protocols. Tissue was either snap frozen for subsequent isolation of genomic DNA or immediately fixed in formalin. The quality of tissue provided for these procedures was considered to be optimal.

4.4.2. Histological analysis of patient pancreas

To confirm a pattern of insulitis and islet destruction consistent with the clinical diagnosis of IDDM

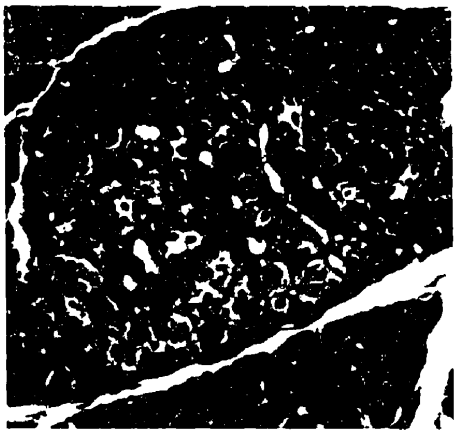
and to assess the severity of damage histological analysis of paraffin embedded pancreatic tissue was carried out. The average insulitis score from 30 islets was determined to be ~1.8. Insulitis lesions were graded as follows: 0 = normal islet, 1 = islet mononuclear cell infiltration of <25%, 2 = 25-50% islet infiltration, 3 = >50% islet infiltration, 4 = reduced islet with little mononuclear cell infiltration. A representative islet was stained with hematoxylin and eosin, and with a panel of immunological stains (Figure 24).

4.4.3. Detection of reverse transcribed CB4 cDNA in the pancreata of a recently deceased IDDM patient

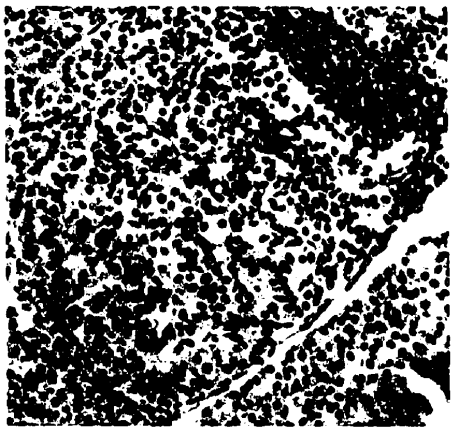
The virus most often associated with human IDDM is CB4. This virus belongs to the same family of viruses as EMC-D and it has been reported that it is capable of cytolytic destruction of human pancreatic β-cells in a manner similar to that of EMC-D in mice (Yoon and Kominek, 1996). For these reasons we chose to examine pancreatic tissue from a recently deceased IDDM patient for expression of a cDNA form of CB4. PCR analysis of a single genomic DNA

Figure 24. Histological analysis of human pancreas.

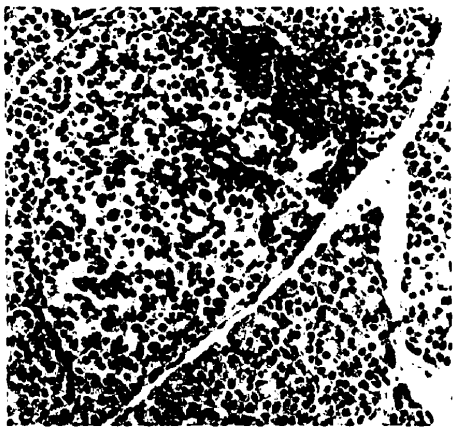
H + E



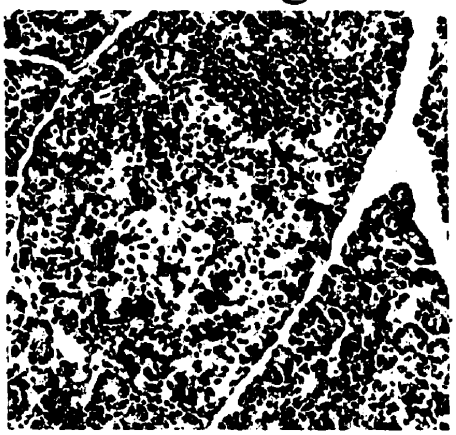
α- CD4



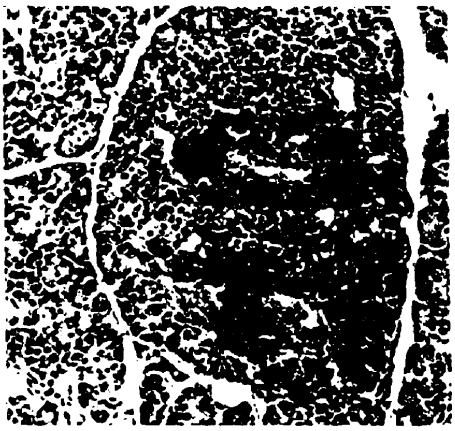
α- CD8



Glucagon



α- insulin

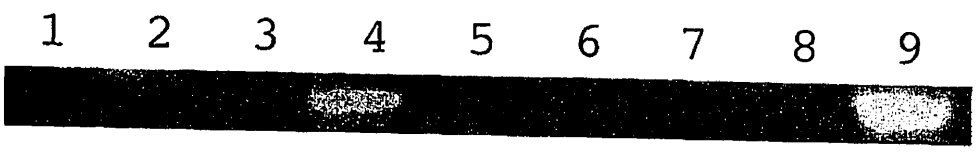


isolate with CB4 E2 VP1 specific primers revealed a single positive signal for CB4 (Figure 25).

4.4.4. Discussion

Access to a pancreatic tissue sample from a recently deceased diabetic patient is a rare occurrence. The value of such a sample is great. We were able to confirm islet pathology consistent with IDDM in paraffin embedded pancreatic tissue. A positive PCR signal was observed in a single genomic DNA isolate of the 17 isolates analyzed to date. The implication that favours a role for CB4 cDNA in the etiology of human IDDM is that detection of cDNA is difficult because most of islet cells harboring the virus have already been eliminated. Though kinetics of diabetes onset in mice indicates that cDNA should be detectable at the onset of clinical diabetes (Figure 11 & 12) the pattern of progression in humans may differ. Ex) the onset of EMC-D induced diabetes in DBA/2 mice is sudden; a matter of days p.i.. Very little is known regarding the prediabetic period subsequent to infection with a diabetogenic virus in humans. In addition the time of actual onset of diabetes can differ significantly from the time of diagnosis. If the

Figure 25. Detection of CB4 cDNA in a single genomic DNA isolate from a recently deceased IDDM patient. Genomic DNA was isolated from ~50mg slivers of frozen pancreatic tissue (Lanes 1-8). Standard PCR was conducted on the isolated genomic DNA using CB4 E2 specific primers. 9: $1:10^2$ CB4 RT product positive control.


1
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9

pattern seen in EMC-D infected DBA/2 mice holds true a matter of weeks is sufficient for a loss of ~50% in PCR positivity (Figure 12C).

5. Overall Discussion and Future Perspectives

5.1. Overall Discussion

The aim of this study was to examine the effects of persistent viral infection, either in the form of permanently integrated endogenous retroviruses or in the form of reverse transcribed cDNA subsequent to exogenous infection by the non-retroviral RNA viruses EMC-D in mice and CB4 in humans.

We were able to identify a unique retroviral sequence from a MIN6N8a cell line derived cDNA library (Table 5) by screening isolated plaques with prediabetic NOD mouse sera. It was demonstrated that the NOD mouse was capable of generating a humoral response against a fragment of the envelope protein encoded by the smallest of our clones, 3-2. This envelope protein fragment was not, however, capable of inducing a cell mediated proliferative response of NOD T-cells. The lack of a cell-mediated immune response against our envelope protein limits its potential significance as a triggering autoantigen.

The relevance of another retroviral sequence, this time in humans, was examined. In follow up to a publication in which a retroviral sequence, IDDMK$_{1,2}$22, was identified as being uniquely expressed in IDDM patients but not normal

controls we endeavored to confirm the expression of this sequence before undertaking more extensive studies on the role of this newly characterized endogenous retrovirus. The new retrovirus possessed homology with superantigen sequences and as such this work had the potential to become a seminal work in he area. Unfortunately we produced results in diametric contradiction to those previously published but our contention that the expression of the new endogenous retroviral sequence was ubiquitous (Kim et al., 1999) was independently confirmed by other groups (Badenhoop et al., 1999; Jaeckel et al., 1999; Muir et al., 1999).

Having been unable to discover a direct role for endogenous retroviruses in the cell-mediated pathogenesis of IDDM we next examined persistence of other RNA viruses via a novel mechanism; reverse transcription in trans by reverse transcriptase encoded by endogenous retroviruses on non-native viral RNA genomes. We were able to detect maximal expression of a cDNA form of EMC-D at 1 week post infection from mice in an experimental situation and CB4 cDNA in a single genomic isolate from a recently deceased human diabetic. We were able to delay the progression to

diabetes in EMC-D infected DBA/2 mice by oral administration with AZT.

Better than two-thirds of the viruses implicated in IDDM pathogenesis are RNA viruses (Table 5). The in trans reverse transcription phenomenon has been encountered previously (Klenerman et al., 1997; Zhdanov, 1975) and is reportedly effective in generating cDNA from the viral RNA genomes of LCMV, measles virus, sindbis virus, and tick-borne encephalitis virus (TBEV). LCMV cDNA has been detected both in vitro and in vivo while the phenomenon has only been detected in vitro with the latter viruses. Of the listed viruses, measles virus has been positively associated with IDDM (Notkins, 1977). The possibility of a single unifying factor in the etiological pathway leading to IDDM bears considerable thought.

Evidence for elevated expression of endogenous retroviruses in mouse strains exhibiting spontaneous autoimmune syndromes and antibody reactivity to retroviral proteins in humans with autoimmune syndromes have convinced many that endogenous retrovirus could play a direct role in the etiology of autoimmune diseases (Stoye, 1999). The body of epidemiological data is, however, often contrary and a definitive association between endogenous retroviruses and

any autoimmune disease has yet to be established (Lower, 1999; Nakagawa and Harrison, 1996; Stoye, 1999). It has been more convincingly shown in some studies that autoimmune phenomena do not genetically segregate with endogenous retroviral expression (Datta et al., 1982; Vyse et al., 1996). The persistence of other RNA viruses due to the action of endogenously encoded reverse transcriptase has not, until now, been examined in the context of and autoimmune disease.

5.2. Future Perspectives

Though an effect was observed, treatment with AZT was neither successful in the complete prevention of diabetes nor even in the prevention of in vivo cDNA formation. Future studies should focus on the complete prevention of cDNA formation. There are at present two major classes of antiretrovirals: reverse transcriptase inhibitors and protease inhibitors. The latter are presently thought to be of little consequence for our purposes but a variety of different RT inhibitors aside from AZT are available and should be considered. Alternate testing of individual, or combinations of, RT inhibitors such as didanosine (ddI),

lamivudine (3TC), stavudine (d4T), zalcitabine (ddC), delvaridine, and nevirapine may demonstrate greater efficacy than AZT in the prevention of EMC-D virus cDNA formation.

A more laborious, but potentially more effective, approach would be the generation of transgenic mice expressing antisense sequences specific for the highly conserved pol gene, which encodes retroviral reverse transcriptase. Antisense therapies have been previously considered as a means of demonstrating the relevance of retroviral expression in diabetes pathogenesis. Such an approach was handicapped by the perceived necessity of identifying the particular retrovirus involved. To date it has not been possible to definitively identify the retrovirus(es) concerned. Our hypothesis, underscoring the importance of retroviral reverse transcriptase, obviates the necessity of targeting a specific retrovirus because of the dependence of the antisense construct design on the most highly conserved retroviral gene.

Our studies have focused on the two best candidates for viral induction of diabetes, EMC-D virus and CB4 virus in mice and humans respectively. Future studies should

examine the ability of other RNA viruses implicated in IDDM to persist via a cDNA intermediate.

Our contention is that the immune system is involved in the eventual destruction of β-cells in mice infected with a low, non-cytolytic, dose of EMC-D. In furtherance of such a stance we feel that determination of chemokine/cytokine, i.e. IFN-α, levels following experimental infection of virus should be examined.

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