Peptide Dose, MHC Affinity, and Target Self-Antigen Expression are Critical for Effective Immunotherapy of Nonobese Diabetic Mouse Prediabetes

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

Cross-reactive T cells which recognize both. Tep69- and ABBOS-epitopes in the islet autoantigen, ICA69 and bovine serum albumin (BSA), are routinely generated during human and nonobese diabetic (NOD) mouse prediabetes. Here we analyzed how systemic administration of these peptides affects progressive autoimmunity in adoptively transferred and cyclophosphamide-accelerated NOD mouse diabetes. Unexpectedly, high dose i.v. ABBOS prevented, while Tep69 exacerbated, disease in both models. Peptide effects required cognate recognition of the endogenous self-antigen. The affinity of ABBOS for NOD I-A^d was significantly higher than that of Tep69. This explained 1) the expansion of the ABBOS and Tep69 T cell pools following i.v. Tep69, 2) long term unresponsiveness of these cells after i.v. ABBOS, and 3) precipitation of disease after low dose i.v. ABBOS. ABBOS or ABBOS-analogs with even higher MHC-affinity may be candidates for experimental intervention strategies in human prediabetes, but the dose translation from NOD mice to humans requires caution.
Contributors

The data presented in the thesis have been published in the Journal of Immunology:


Each author made the following contribution to the paper:

Shawn Winer: I was in overall command of the project and performed and analyzed the bulk of the data, including Figures 1-5, 7-9. The data described in Figure 1 was gathered during my fourth year undergraduate research project, when I worked with a former MSc. student of the Immunology department, Lakshman Gunaratnam.

Lakshman Gunaratnam did the initial adoptive transfer experiments, which I reproduced and expanded. As a summer student he contributed to data in Figures 1, 2A-B, 3A, 7:

Igor Astsatourov did all of the MHC binding experiments (Figure 6);

Roger Gaedigk, Violetta Kubiak, Wolfram Karges, Roy K. Cheung, and Denise Hammond-McKibben developed the ICA69null 129-line mice and generated the NOD congenic ICA69 knockout;

Daniel Graziano, Massimo Trucco: taught us MHC binding assays and provided reagents;

Dorothy J. Becker: intellectual input.
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Abbreviations

aa: Amino acid

ABBOS: An immunodominant epitope in BSA

ALA: Alanine

APC: Antigen Presenting Cell

BB: BioBreeding

BCG: Bacillus Calmette-Guerin

BSA: Bovine Serum Albumin

CFA: Complete Freund’s Adjuvant

CY: Cyclophosphamide

DPT-1: Diabetes Prevention Trial 1

EBV: Epstein-Barr Virus

FDR: First Degree Relative

FITC: Fluoroscein Isothiocyanate

GAD65/67: Glutamic Acid Decarboxylase (65 or 67 kD isoform)

HPLC: High Performance Liquid Chromatography

HLA: Human Leukocyte Antigen

HSP60: Heat Shock Protein (60 kD)

I-A2: A protein tyrosine-phosphatase

I-A^7: MHC class II molecule in NOD mice

ICA: Islet Cell Antibodies

ICA69: Islet Cell Antigen (69 kD)

IDD(M): Insulin Dependent Diabetes (Mellitus)
IFA: Incomplete Freund’s Adjuvant

IFN-γ: Interferon Gamma

IL-: Interleukin-

i.p.: Intraperitoneal

i.v.: Intravenous

kD: Kilodalton

MHC: Major Histocompatibility Complex

MBP: Myelin Basic Protein

MS: Multiple Sclerosis

NK: Natural Killer

NOD: Non-Obese Diabetic

NON: Non-Obese Nondiabetic

NOR: Non-Obese Diabetes Resistant

PBS: Phosphate Buffered Saline

PPD: Purified Protein Derivative

RT-PCR: Reverse Transcription Polymerase Chain Reaction

SCID: Severe Combined Immunodeficient

TCR: T Cell Receptor

Tep69: T Cell Epitope 69 kD

Th: T Helper

TNF: Tumour Necrosis Factor

TRIGR: Trial to Reduce Diabetes in the Genetically at Risk

VNTR: Variable Number of Tandem Repeats
I. Introduction

I.1. Autoimmune Diabetes: A Clinical Perspective

Insulin dependent or Type I diabetes mellitus (IDDM or T1DM) or autoimmune diabetes is a chronic disorder that results from lymphocyte mediated destruction of the pancreatic beta cells in the islets of Langerhans (1). Disease onset is characterized by hyperglycemia, ketoacidosis, frequent urination, thirst, dehydration, and weight loss: all symptoms associated with insulin deficiency (2). The current treatment for diabetes is insulin replacement therapy.

Although daily insulin injections provide an effective treatment for autoimmune diabetes, numerous micro- and macro-vascular disease-related complications develop later in life. For example, twenty years after diagnosis, 40% of diabetic patients will be affected by neuropathy, 48% by retinopathy and 10% by nephropathy (2). Accordingly, autoimmune diabetes is the leading cause of blindness and end-stage renal disease (1). The overall life expectancy of diabetic patients is reduced by 25%, with those diagnosed earlier in life carrying a higher mortality rate (3). Complication related mortality in diabetic patients is primarily a result of nephropathy (55% of deaths) and cardiovascular disease (15% of deaths) (4).

Diabetes is also a major economic concern in national health care systems (5, 6). It has been estimated that the costs of chronic care and medical support exceeds 124 billion dollars annually in the United States (7, 8). The Canadian Health Minister, A. Rock, singled out diabetes in his announcement of the new Canadian Institutes For Health Research (June 2000) and
reported that overall diabetes costs are 25 Billion dollars annually in Canada; about 80% of these costs go towards T1DM. Thus, prevention/intervention of diabetes or a curative treatment for diabetes would have tremendous impact on patients, families and health care systems.

I.II. Epidemiology

Autoimmune diabetes is a complex, polygenic disorder in which genes and environment act conjointly in disease development (1). The disease affects at least 0.5% of Caucasians, but varies dramatically between different ethnic groups and countries (9). For example, the diabetes incidence ranges from a low of 1 to 2 per 100,000 in Japan to a high of now 50 per 100,000/year in Finland (10). There is a pronounced north-south gradient for diabetes development, with higher incidences occurring more frequently in northern countries (11). Differences in global diabetes incidence have tentatively been explained by a higher prevalence of T1DM susceptibility genes within a population and by environmental factors (12). The importance of environmental factors is supported largely by the low (30%) twin concordance (13-15), north-south gradient, migration studies (9, 16-18), and the rapid global rise in autoimmune diabetes incidence over the last 40 years (19, 20).

Among various environmental factors, infectious agents and nutrition have been associated with T1DM risk (1). Exposure to infectious agents and their putative superantigens (21, 22), rubella (23), the Coxsackie B viruses (24-29) or other enteroviruses have been implicated in the pathogenesis of autoimmune diabetes, but definitive links are lacking, and the
mechanisms remain unclear and controversial (30). Nutritional factors such as a high intake of nitrosamines (31, 32) and an early exposure to cow's milk-based infant formulas (33) have been associated with high diabetes risk (discussed below), while exposure to the vitamin B derivative, nicotinamide (34–44), and vitamin D3 (an IL-12 antagonist) have been associated with diabetes protection (45–48).

Unlike multiple sclerosis, rheumatoid arthritis, and several other autoimmune disorders, diabetes develops equally among males and females. Type I diabetes can develop at any age in life (49), with disease uncommon near birth and peaking in incidence at puberty (50, 51). Although diabetes occurs most frequently before the age of twenty, there is also a second peak in incidence, occurring much later during adult or mid-life age (52). Autoimmune diabetes in older patients has a reduced prevalence of ketoacidosis at diagnosis, and a better preservation of residual beta cell function (53). Common among Japanese patients living in Japan (54), but not elsewhere (55), this form of Type I diabetes is most closely reminiscent of the disease in non-obese diabetic (NOD) mice (56). It is not known what triggers diabetes, and how or why diabetes requires many years to develop (1). These are some of the fundamental questions in the field.

I.III. Genetics of Human T1DM

While nearly 20 chromosomal regions have been associated with T1DM susceptibility, a large part of the genetic risk (>60%) maps to the MHC class II on chromosome six (57). A small number of common HLA-DR and HLA-DQ alleles determine T1DM susceptibility or resistance.
(58). HLA-DR3-DQ8 and HLA-DR4-DQ2 are strong predictors of T1DM development (59). Ninety percent of diabetic Caucasians possess the DR3 and/or the DR4 allele, with heterozygosity for these alleles carrying the highest risk (60). Homozygosity in the HLA-DQ8 allele, which contains a non-aspartic acid at position 57, usually valine, serine or alanine, mediates the highest diabetes risk (61-64). The absence of the negatively charged aspartic acid residue at position 57 has been suggested to reduce the stability of peptide-HLA-DQ complexes, resulting in a generally low MHC affinity for peptides (65). Intriguingly, like the HLA-DQ8 molecule, HLA-DR3 and HLA-DR4 also bind diabetes associated peptides with very low affinity (66). It has been proposed that a reduced peptide binding affinity to MHC II may allow autoreactive T cells with high affinity TCR's, to escape negative selection and enter the periphery, where they might participate in diabetes development (67-69).

HLA-DR2 (DR15)-DQ6 and HLA-DR11-DQ5, are associated with disease resistance (70, 71). HLA-DR2 has a dominant protective effect since individuals carrying HLA-DR2 are resistant to diabetes even if they are carrying one of the high risk HLA molecules such as HLA-DR4 or HLA-DR3 (1). HLA-DR2 binds diabetes associated peptides with very high average affinities (66). High affinity binding might delete potentially diabetogenic T cells during negative selection, thereby contributing to disease resistance. However, DR2 and its associated DQ6 are linked to the risk of developing multiple sclerosis (MS) (72): diabetes and MS-associated MHC class II molecules thus appear to lie at opposite ends of the spectrum of average MHC peptide-binding affinities, both permissive (and perhaps prerequisite) for the development of pathogenic T cell autoreactivities (73).
Although HLA-DR3, HLA-DR4, HLA-DQ8 and HLA-DQ2 alleles are associated with disease susceptibility, these alleles are commonly found in normal, unaffected individuals. This emphasizes the importance of other T1DM susceptibility genes in the development of diabetes (74). Few of the non-MHC susceptibility genes/protein products are well defined, but major efforts are dedicated towards their characterization (75). For example, one major non-MHC susceptibility locus in humans (Iddm2) maps to the variable number of tandem repeat (VNTR) polymorphisms in the 5' flanking region of the insulin gene (76). The exact role of this polymorphism in diabetes etiology is unknown, but was suggested to somehow affect transcription levels of the insulin gene possibly in the thymus (77-84).

Interestingly, the genetic location of some of the non-MHC susceptibility loci overlap with loci from other autoimmune diseases, suggesting shared or common defects underlying autoimmunity (85, 86). For example Iddm2 overlaps with loci linked to systemic lupus erythematosus (SLE), ankylosing spondylitis, asthma, and multiple sclerosis. Identifying the gene products associated with diabetes susceptibility loci would greatly enhance our understanding of the mechanisms underlying diabetes and other autoimmune disorders.

I.IV. The NOD Mouse: A Brief Introduction

The non-obese diabetic (NOD) mouse was discovered some twenty years ago in Japan (87). Since then, it has become the most widely studied animal model for autoimmune diabetes. The NOD mouse develops autoimmune diabetes spontaneously, and like humans, its disease development is dependent on both environmental and genetic factors. The similarities between NOD and human diabetes are striking (56). For example, NOD mice target a similar, possibly
even identical set of autoantigens as human diabetic patients (discussed below). Furthermore, analogous to human diabetes, a large part of the genetic risk in the NOD mouse maps to the MHC class II locus (88, 89). The NOD mouse does not express the DR-equivalent, MHC class II I-E molecules due to a mutation in the Eα promotor region (90). The NOD mouse therefore expresses only one (DQ-equivalent) MHC class II molecule, the unique I-A^87, which contains a non-aspartic acid substitution (serine) at position 57 in the β chain, much like the human HLA-DQ8 (58, 90). Similar to human HLA-DQ8, the NOD I-A^87 has also been reported to be a poor peptide binder, which may reduce the efficiency of negative selection and lead to an excess of high affinity autoreactive T cells in the periphery (67, 91). In the MHC class I locus, the NOD mouse contains the K^d and D^b alleles, which are not associated with spontaneous autoimmunity (90).

NOD mice, congenic for the C57BL/6 MHC II, H-2b, fail to develop severe invasive insulitis, cyclophosphamide induced diabetes, or spontaneous diabetes, highlighting the importance of MHC class II in disease development (92). Although MHC class II is critical for disease, it is not sufficient to induce disease by itself (93, 94). This idea was demonstrated by the absence of diabetes development in congenic mouse lines such as the NOR, NON, and C57BL/6 H-2^87 strains (92, 93, 95, 96).

Like human diabetes, close to twenty non-MHC genetic loci have been implicated in NOD diabetes (75). Although no non-MHC susceptibility gene has been definitively identified, sequence variation in the structural gene for IL2 has been suggested to define the Idd3 locus (97), and candidate genes have been identified for several other loci (98-101).
In addition to autoimmune diabetes, and similar to the human condition, the NOD mouse develops features of other autoimmune diseases (56). For instance, older NOD mice show signs of mild Sjögren's syndrome, with mononuclear cell infiltration in the submandibular/salivary and lacrimal glands (102). A high frequency of mild spontaneous thyroiditis has also been observed in NOD mice (103). In my research subsequent to the present thesis work, I demonstrated that NOD mice also develop autoimmunity to myelin-associated antigens, but fail to develop spontaneous central nervous system disease, possibly due to a tight blood brain barrier (73). Like NOD mice, human diabetic patients also display a higher frequency of susceptibility to other autoimmune diseases, such as Hashimoto's thyroiditis (104). Susceptibility to multiple autoimmune diseases for diabetic patients and mice is likely due to the overlap of susceptibility loci among different autoimmune diseases (85, 86).

Since much of the contemporary knowledge of type 1 diabetes has been derived from studies utilizing the NOD mouse, this thesis will focus primarily on NOD mouse diabetes. Although NOD mouse diabetes exhibits a strong resemblance to human diabetes, there are several differences. For example, the NOD mouse displays a robust gender bias with females developing disease approximately 2-3 times more likely than males (105), and NOD mice are relatively resistant to ketoacidosis as compared to most human patients (56). These characteristics plus the relatively late disease onset are typical for T1DM in Japanese patients living in Japan. Collectively, the many similarities between NOD and human diabetes has made the NOD mouse the ideal model to study diabetes pathogenesis (56).
I.V. Pathogenesis

NOD mouse (and likely human) diabetes develops in discrete stages (106). Autoreactive T cells are detectable within two weeks of weaning, and peri-insulitis is slowly established over the next several weeks of prediabetes (107). Mid to late stage prediabetes is characterized by the progression to invasive insulitis, which is prominent by 2-3 months, and signals the beginning of significant islet destruction with consequent autoantibody production (108). The pace of beta-cell destruction peaks close to overt disease, at ages 5-7 months (109). Symptoms of diabetes, including hyperglycemia and ketosis, develop when approximately 80-90% of the beta cells have been destroyed. Beta cell death is thought to occur acutely and relatively late in the disease process (109), rather than gradually over a long period of time. However, the issue of the time of beta cell death during diabetes is not fully settled (110). Only beta cells are eradicated in diabetes; alpha cells (glucagon secreting cells) and delta cells (somatostatin secreting cells) are preserved, demonstrating the exquisite beta cell specificity of autoimmunity in diabetes.

The inflammatory infiltrate in the islet consists of CD8+ T cells, CD4+ T cells, B cells, macrophages and NK cells (111, 112). Both CD8+ and CD4+ T cells are critical for disease development. This was demonstrated by the absence of diabetes development in studies using anti-CD4 (113) or anti-CD8 (114) antibodies. Studies using MHC class I and MHC class II deficient mice obtained similar results (115, 116), confirming the importance of both CD4 and CD8 T cells for disease development. It is believed that CD8+ T cells participate in all earlier stages of diabetes development (114, 117), possibly in the initiation of disease via cytotoxic effector mechanisms (118). CD4+ T cells are thought to be especially important for the
progression and end stages of disease. However, the exact roles of CD4+ and CD8+ T cells remains unknown (119). Antigen presenting cells are also critical for disease as indicated by the reduction or absence of diabetes in B cell deficient mice (120, 121) and macrophages/dendritic cell depleted NOD mice (122, 123).

During the course of insulitis, T cell autoimmunity to several proteins is established (124, 125). CD8+ T cell reactivity to proinsulin has been observed in the infiltrates of NOD islets at the onset of insulitis (4-5 weeks), but are reduced in frequency in islets of older mice (118). CD4+ T cells have been shown to recognize various proteins including GAD65, GAD67, (pro)insulin, hsp60, I-A2/phogrin, and ICA69 (1). With the exception of insulin, none of these target proteins are beta cell specific.

It has been suggested by some (124, 125) but not other groups (118), that loss of T cell tolerance occurs first to GAD65 at the beginning of insulitis. With subsequent tissue damage, diabetic autoimmunity spreads to other islet cell proteins by a process known as intermolecular determinant spreading (124, 125). Spreading of autoimmunity to different epitopes within a protein also occurs during diabetes development by a process known as intramolecular determinant spreading (126, 127). The emergence of new epitopes in GAD65 is one example of intramolecular determinant spreading (127). An exception is presented by ICA69, where no epitope spreading has been observed, and patients as well as NOD mice target solely the conserved ‘Tep69’ epitope (64, 128-130). Both intermolecular and intramolecular spreading contribute to the complexity of disease development and may pose obstacles for possible antigen specific immunotherapeutic intervention strategies (131).
The progression of diabetes is associated with an antigen specific T helper 1 (Th1) bias in CD4+ T cell cytokine profiles (132). Th1 biases are associated with cellular immunity and the production of pro-inflammatory cytokines like TNF-beta, IL2, IL12, and IFN-γ. A role for IFN-γ in diabetes development was suggested since transgenic expression of IFN-γ in the islets can induce diabetes in BALB/c mice (133). IFN-γ acts by enhancing macrophage activation and in the induction of MHC class I and class II overexpression, leading to more efficient antigen presentation (134). The development of a Th1 bias during diabetes suggests that a pro-inflammatory cascade may drive disease progression (132). In contrast, Th2 biases, which are associated with humoral immunity and secretion of IL-4, IL-5 and IL-10, are thought to be protective against diabetes development. The issue, however, is not resolved since i) diabetes develops normally in IFN-γ and IL-12 deficient NOD mice (135, 136), ii) diabetes develops in mice with islets transgenic for IL-10 (137), iii) diabetes is not exacerbated in IL-4 deficient NOD mice (138) and iv) Th2 T cell clones from a CD4+ TCR transgenic mouse can cause diabetes in NOD.scid recipients (139). It is uncertain whether cytokine Th1 bias is the cause or the result of tissue damage.

At the onset of disease, autoantibodies to insulin, ICA, ICA69, GAD65, and IA2 are abundant in diabetic patients (140) and NOD mice (141). Autoantibodies to several of these proteins are now used in relatives of index cases as increasingly reliable markers of disease risk, in particular when analyzed in conjunction with MHC class II genotypes (52, 63, 142-152). The ability to reliably (~90%) predict the presence of progressive prediabetes up to 15 years prior to disease onset (153) has become the cornerstone of diabetes intervention efforts such as the $34
Million U.S. insulin trial effort, DPT-1 (42, 60). Research described in this thesis is the type of study required as a basis for future human interventions, which would likely recruit adult pre-diabetics headed towards late-onset Type I diabetes (63, 64, 153).

The role of autoantibodies in the pathogenesis of autoimmune diabetes is unclear, and most likely reflects the presence of beta cell destruction (108, 141). Unfortunately, human disease staging is still inadequate, as there is virtually no tissue access to identify the degree of progression through analysis of insulitis (64, 154-156). The use of multiple autoantibodies as risk markers to identify individuals for disease intervention trials almost certainly identifies subjects where beta-cell destruction is already well established. While immunotherapy of NOD mice has been demonstrated to be highly efficient when applied early, prior to the beginning of invasive insulitis, very few manipulations are effective when administered late (reviewed in (56), discussed below). This is the rationale for work in this thesis, which focuses on determining mechanisms of immunotherapy, using models of mid-late stage prediabetes. The use of multiple T cell autoreactivities as a risk marker, may improve prediabetes staging, as the development of T cell autoreactivity to recognized ‘diabetes autoantigens’ appears to precede autoantibody production in humans (64) as well as NOD mice (124, 157).

I.VI. Cow Milk and Diabetes: Does Bovine Serum Albumin Play a Role?

Well over fifty studies have linked exposure to cow’s milk during early infancy and/or a lack of breast-feeding as risk factors for the development of T1DM (ref: 1, 158-161). In two particularly significant studies, which controlled for MHC risk alleles, the relative diabetes risk
for early weaning to cow's milk-based formula was 11-13.1, more than the risk associated with cigarette smoking and lung cancer (relative risks between 6-8) (159, 162). Based on these findings, the American Academy of Pediatrics has recommended breast feeding, and a delayed use of cow’s milk based formula in infant’s with a family history of T1DM (163).

Additional evidence for a role of cow milk in diabetes development comes from studies in diabetes-prone rodents. NOD mice which are weaned to a fully hydrolyzed protein-free formula (Nutramigen™) are robustly protected from diabetes (5-10% diabetes incidence by 45 weeks of age) (164). A world-wide diabetes prevention trial, the "Trial to Reduce IDDM in the Genetically at Risk" (TRIGR), tests whether the robust data from diabetes-prone BB rats and NOD mice can be translated to newborn infants with genetic diabetes risk (42). The Dosch laboratory is closely involved with the development and conduct of this trial, which is moving to its international phase after nationwide Finnish pilot studies had promising results (165).

The mechanisms by which cow’s milk exposure may precipitate diabetes are controversial and not well understood (166-170). Dosch and co-workers, in Toronto, favor an immunological basis for this correlation (129, 164, 171). Previously, they have demonstrated that diabetic patients have elevated titres of antibodies against bovine serum albumin (BSA), a cow’s milk protein, relative to control, non-diabetic individuals (171-173). Anti-BSA antibodies were shown to cross-react and precipitate a 69 kD pancreatic beta cell protein, islet cell antigen 69 (ICA69) which they cloned, characterized and targeted in knock-out experiments in C.elegans and mice (128, 130). Elevated BSA and ICA69 T cell responses are common in newly diabetic patients, their high-risk relatives and in diabetic NOD mice (64, 129, 130).
ICA69 is a neuroendocrine molecule with peak expression in pancreatic beta cells, brain and testes (174). The protein contains no sequence homology with other known proteins, is largely cytoplasmic, but is also localized to vesicles (174). Although the function of ICA69 is not known, knock-out experiments in *C. elegans* suggested that the protein may participate in neurotransmission (175).

By comparing homologous sequences between BSA and ICA69, Dosch and co-workers mapped the immunodominant epitope in BSA to the ABBOS peptide (BSA-150-164), and the immunodominant epitope in ICA69 to the Tep69 peptide (ICA69-36-47) (128, 130) (Table 1).

### Table 1. Linear Sequence Homology Between ABBOS and Tep69 Peptides

| (ABBOS) BSA-150-164: | FKADEKKFWGKYLYE |
| (Tep69) ICA69-36-47: | AFIKATGKKED |

Several different length variants of the ABBOS peptide (including the BSA-147 variant used in figure 1C.D of this study) and of Tep69 peptide variants (including the ICA69-35 variant used in figure 1C.D of this study) were shown to be equally efficient in inducing proliferative responses in diabetic patients (130). However, identification of a functional, minimal peptide for ABBOS or Tep69 has not yet been concluded. The ABBOS region of BSA is poorly conserved among mammalian albumins, including human, goat and horse serum albumin, due to a recent sequence mutation (176, 177).
ABBOS and Tep69 share the common KAxyKK amino acid motif. Immunization of NOD mice with either BSA/ABBOS or ICA69/Tep69 induces cross-reactive T cell responses (129). BSA and ICA69 share two other regions with even greater structural homology than ABBOS and Tep69, but these regions fail to elicit mimicry responses in diabetic patients or NOD mice (129, 130). Thus, the presence of a high degree of linear sequence homology between two peptides does not necessarily indicate the presence of mimicry.

Tep69 and ABBOS specific T cells have been shown to be significant contributors in diabetogenesis. Neonatal tolerization of NOD mice with Tep69 protects from diabetes (129), while treatment of young mice with ABBOS in IFA reduces the diabetes incidence. In a recent study, the presence of concordant ABBOS and Tep69 responses was found to be significantly higher in diabetic patients and high risk first degree relatives compared to low risk first degree relatives, who tend to respond to either Tep69 or ABBOS, but not to both (p=0.001 and p=0.012, respectively) (64). Therefore, the presence of immunity to both ABBOS and Tep69 appears to be a marker for disease risk.

A molecular mimicry model for the pathogenesis of diabetes was thus proposed. In this model, early infant exposure to dietary BSA/ABBOS would rescue ICA69/Tep69 specific T cells from tolerance mechanisms, thereby generating and sustaining autoimmune cells at numerically significant levels. A goal of this thesis will be to provide more insight into the validity of this model, and to substantiate the possible significance of ABBOS and Tep69 immunity during diabetes development.
I.VII. Antigen-Based Immunotherapy

Immunotherapy with autoreactive proteins or peptides has been a very successful means in preventing autoimmune diseases in NOD mice (reviewed in (178)). Immunotherapy is an attractive intervention strategy in comparison to immunosuppressive approaches, since it tolerizes (157, 179, 180), inactivates or deviates (181, 182) autoreactive T cell functions without interfering with the remaining T cell pool. Various routes of administration of autoantigen were demonstrated to be effective for antigen specific interventions, including subcutaneous or intraperitoneal administration in incomplete Freund's adjuvant (IFA), intranasal, intravenous (i.v.), intrathymic and oral antigen delivery (178). In NOD mouse experiments, early treatment with any one of the diabetes-associated autoantigens has been shown to reduce the incidence of the disease. For example, intranasal, oral, intravenous and subcutaneous (in IFA) insulin treatments of 4-5 week old NOD mice prevented diabetes in all reports (183-185). This was one rationale behind the creation of the first human diabetes prevention trial (DPT-1), which focuses on prevention of diabetes development using insulin. This trial is now in progress, and utilizes subjects who are recruited from high risk relatives of index cases, arrived, however, at a very late stage of prediabetes.

In animal models, most immunotherapies, however, are successful only when employed early in prediabetes, before the onset of established autoimmunity (i.e. prior to the invasive insulitis phase of prediabetes) (56). For example, GAD65 derived peptides p217, p290 and p247/p524, when emulsified in IFA and given to 4 week old NOD mice (pre-/ early peri-insulitis) all protected from diabetes. However, when administered to 12 week old NOD mice (invasive insulitis), all peptides failed to prevent disease (186). Understanding the mechanisms of immunotherapy in late stage prediabetes is essential and an important goal for diabetic
immunotherapy since human recruits into intervention trials are identified through measurements of often multiple autoantibodies, i.e. relatively late in prediabetes.

Effective immunotherapy during established autoimmunity can in principle be successful. GAD65 protein, emulsified in IFA, was shown to prevent diabetes when administered to 8 week old NOD mice and prevent adoptively transferred diabetes (187). Disease protection was associated with an overall shift towards a Th2 phenotype in diabetic T cell responses. In another study, a high affinity analog of the immunodominant myelin basic protein (MBP) peptide Ac1-11 was shown to prevent experimental autoimmune encephalitis (EAE) in (PLJxSJL)F1 mice after the onset of relapses (188). The proposed mechanisms for the treatment include clonal deletion and a shift towards a Th2 cytokine profile. However, the fundamental mechanisms dictating effective versus ineffective immunotherapy during established autoimmune disease are poorly understood and require further study, including determination of causality for helper T cell biases.

II. Study Objectives

The primary objective of this thesis was to examine the therapeutic potential of the ABBOS and Tep69 peptides in NOD mice, using experimental models for established and accelerated autoimmune disease. These models of diabetes were adoptive transfer (189) and cyclophosphamide accelerated disease (190). Both models were chosen because of their close resemblance to the later stages of prediabetes.

There were two secondary objectives for this thesis.
1.) To determine the mechanisms of ABBOS and Tep69 peptide action. The mechanism was examined by determining the effects of intravenous ABBOS and Tep69 peptides on the T cell response in NOD mice, and by correlating the in vivo peptide effects with the in vitro peptide-MHC affinities.

2.) To analyze these data in the context of the long standing BSA and ICA69 mimicry hypothesis developed by the Dosch lab.

III. Materials and Methods

III.I. Mice

NOD/Lt (H-2k) mice were originally purchased from the Jackson Laboratories (Bar Harbor, ME) and bred and maintained in our conventional rodent facility according to facility guidelines and approved protocols. Our conventional vivarium has a diabetes incidence of 83% in female mice ≥6 months of age. ICA69null mice were generated by homologous recombination in 129-line embryonal stem cells, disrupting the Tep69 coding sequence in exon-2 (191). A speed-congenic approach with published microsatellite markers (120) was employed to develop the animals (6th backcross generation to NOD) with all Idd loci of NOD origin. ICA69null NOD mice have a diabetes incidence comparable to wild-type NOD mice (192).

III.II. Peptides

Peptides were purchased HPLC purified (>98%) and confirmed by mass spectroscopy (numbers indicate the N-terminal amino acid position): Ovalbumin-152, EYQDNRSFLGHFI;
GAD65-524, SRLSKVAPVIKARMMEYGT; HSP60-277, VLGCCALLRCIPALDSLTTPANED. ICA69-36 (Tep69), AFIKATGKKEDE, BSA-150 (ABBOS), FKADEKKFWGKLYE. Grade V BSA and ovalbumin were purchased from Sigma (St. Louis, MO) and the human ICA69-β isoform was purified as described (128).

III.III. E12.3 T cell hybridoma

NOD mice were immunized subcutaneously with 100μg of BSA emulsified in CFA. Ten days later, draining nodes were removed and cultured (3d) in the presence of 5 μg/mL of ABBOS peptide. Cultured cells were fused to BW5147 TCR deficient thymoma cells (ATCC) by the standard polyethylene glycol-fusion protocol. Hybridomas were screened by a cell death assay, which measures the hydrolysis of 2,7-bis-2-carboxyethyl-5-6-carboxyfluorescein (BCECF) dye (Molecular Probes, Eugene, OR) into fluorescent intermediates by viable, but not dying cells (193, 194). Briefly, replicate cultures of 10⁴ E12.3 cells were plated in the presence of 1μg/well of antigen and 10⁴ irradiated (2500 rad) C3G7 B lymphoma cells (stably transfected with I-A*; a kind gift of Dr. E. Unanue) (67) or 2x10⁴ irradiated (2500 rad) spleen cells from NOD or other strains of mice, as indicated in the text. After 72 hours of culture, 8 μg/mL BCECF were added for 45 minutes. Cells were moved to a 96-well filtration plate where free dye was removed through filtration prior to measurement of fluorescent BCECF derivatives in the cytosol of viable cells (IDDEX ScreenMachine, Mundelein, ME). Thus, viable, but not dead cells, display high fluorescence. Data were expressed as relative fluorescence units (RFU), compared to an internal standard (193). This assay provided a rapid, sensitive and highly reproducible alternative to the more common cytokine release assays during the screening for T cell hybridomas described here.
III.IV. Adoptive transfer

Spleen cells from at least four diabetic females were pooled, and transferred i.v. in 100 µl PBS to 7-9 weeks old sublethally irradiated (650 rad) NOD males. Ten x10^6 cells were transferred unless noted otherwise. One day following transfer, recipients were given a single i.v. injection of either 100 µg or 400 µg of peptide in PBS. In all experiments, glucosuria (TesTape. Lily. Toronto. ON) was used to screen for diabetes until 35 days post-transfer. Diabetes was confirmed by blood glucose measurements (SureStep™. Life technologies. Burnaby. BC) (129).

III.V. Cyclophosphamide-accelerated diabetes

Diabetes was induced in 8-12 weeks old NOD females by a single i.p. injection of cyclophosphamide (Sigma. 250 mg/kg if not indicated otherwise). 100 µg of peptide was given i.v. 5 days before cyclophosphamide injection.

III.VI. Histology

35 days after cyclophosphamide treatment, non-diabetic mice were sacrificed and their pancreata were preserved in 10% buffered formalin. Histological sections (4.5 µm thickness) were cut at different levels (100 µm/level) and stained with hematoxylin and eosin. Two blinded observers scored the degree of insulinitis with the following scale: 0-normal islet, 1-periinsulitis or <25% infiltration, 2-infiltration of 25%-50% of islet surface area, 3-infiltration of greater than 50% of islet surface area, 4-complete (100%) infiltration of islet or a small retracted islet.
III.VII. Tolerance induction and proliferative recall assay

Female NOD mice, aged 8-12 weeks, were given a single intravenous injection of usually 100 µg peptide dissolved in PBS. Four or twenty days later, mice were immunized subcutaneously with 100 µg of peptide emulsified in CFA. Lymph nodes were removed 9-10 days after immunization and cultured (4x10⁵ cells/well) in serum-free AIM V medium (Gibco, Mississauga, ON) in the presence of 0.1-10 µg of peptide. On day three, cultures were pulsed with 1 µCi [³H]thymidine overnight, and subjected to liquid scintillation counting. When analyzing T cell responses in animals not formally immunized with CFA emulsified antigen, cells were cultured in the presence of antigen or media and 10U of human rIL-2 which enhanced the amplitudes of positive responses.

III.VIII. I-A<sup>g7</sup> purification and affinity studies

I-A<sup>g7</sup> was purified from lysates of C3G7 cells by affinity chromatography using protein A beads coupled to 10-2.16 Ab (anti-I-A<sup>g7</sup> and anti-I-A<sup>k</sup>) (195, 196). Briefly, 10⁸ C3G7 cells/ml were incubated in lysing buffer (50 mM Na₂HPO₄, pH 7.5, 150 mM NaCl, NP-40 1% (v/v), and protease inhibitors) on ice for 2 hours. Following incubation, the lysate was centrifuged at 27,000g for 30 min, the supernatant removed, and added to cold 5% sodium deoxycholate (DOC, pH 7.5-9.5) for 10 min at 4°C. The resulting solution was spun down (100,000g for 2 hours) and the supernatant filtered through a 0.45µm membrane. The filtered lysate was incubated with the 10-2.16-coated protein A-beads overnight at 4°C and then poured into a column and washed. Bound I-A<sup>g7</sup> was eluted from the column with 50mM diethylamine HCl, pH 11.5, in 0.15M NaCl, 1mM EDTA, 1% octyl-β-D-glucopyranoside (OGP), 10% glycerol, and 0.03% NaN₃, and collected into tubes with 50 µl of 1M Tris, pH 8. SDS-PAGE of the eluted protein revealed only
the α and β chains of murine I-A<sup>87</sup>. The above protocol was adapted from (195).

For the affinity studies, biotinylated peptides at different concentrations were incubated (16 hr, 10<sup>6</sup> cells/ml) with I-A<sup>g7</sup> transfected C3G7 cells at 37°C (67) or DR4/DQB1*0201-homozygous PRIESS cells (197) in 100 µl of complete RPMI-1640, supplemented with 10% horse serum. Cells were then washed and incubated (45 min) with FITC-labeled streptavidin. Cells incubated with FITC-streptavidin alone served as negative controls. After two additional washes, fluorescence was measured by flow cytometry. Relative fluorescence intensity was estimated as mean equivalents of fluorescein (MEFL) with Rainbow Calibration Particles according to the manufacturers instructions (Spherotech, Libertyville, IL). For measurements of dissociation kinetics, C3G7 cells were labeled with peptide as above, washed, and incubated in complete medium for variable periods before labelling with FITC-streptavidin and flow cytometry. In competition studies, unconjugated peptides were incubated during the primary incubation with the biotinylated peptides.

For in vitro affinity studies, binding of biotinylated peptides to purified I-A<sup>g7</sup> (500 ng) was performed in 100 µl of PBS (pH 7.4) and overnight incubation at room temperature. The incubations were transferred to 96-well plates coated with 100 µl purified capture antibodies (10-2.16) overnight and blocked with 10% horse serum-PBS. The peptide-protein mixtures were incubated for 1 hr at room temperature. After four washes (10 mM Tris/140 mM NaCl/0.05% Tween 20, pH 8). Streptavidin-conjugated horse radish peroxidase or alkaline phosphatase was used to detect bound biotinylated peptides in an automated plate reader. A graph of OD (450 nm) versus peptide concentration (µM) was plotted. A Scatchard plot was extrapolated from the graph and data using the following standard equation (198):

$$B/F = \frac{([OD_{max})-(OD)]/[peptide]_{50}}$$
B/F = bound/free ratio

ODmax = the OD (450 nm) at which MHC binding is saturated

[peptide]_{50} = the concentration of peptide that results in 50% saturation of MHC binding

III. IX. Statistics

Numeric data were compared by the Mann-Whitney test. The Mann-Whitney test was used since it makes no assumption that the data fall into a normal distribution. The end-points of the incidence graphs were analyzed by Fisher’s exact test. The Fisher’s exact test was used because it is strict and powerful (gives exact p value) even with small sample sizes. All p values were two-tailed and significance was set at 5%.

IV. Results

IV.I. BSA- and ICA69-specific reactivity in cloned T cell hybridomas

Dosch et al. have previously reported that T cell pools responsive to Tep69 and ABBOS peptides are routinely detectable in NOD mice and patients with recent onset diabetes (64, 128). Immunization of young NOD mice with either peptide (or with either of the native proteins) raises T cell responses to both peptides (129). To analyze T cell mimicry at a clonal level, and to determine if cross-reactive T cells constituted a minor or major proportion of ICA69- and BSA-specific T cell pools, we have cloned a panel of Tep69/ABBOS-specific T cell hybridomas from BSA-immunized NOD mice. Four clones, E12.2.-3, 4 and -5 were obtained. Each clone behaved similarly in vitro: data are shown for E12.3.
E12.3 cells were exposed to antigens/peptides in the presence I-A^87 transfected C3G7 lymphoma cells (67) as APC. Cognate recognition of BSA, recombinant ICA69-β, ABBOS, Tep69 (Fig. 1A), but not Ovalbumin, or OVA152 peptide (Fig. 1B) produced rapid activation-induced cell death as measured in an automated, fluorescence-based viability assay (see Methods for details) (Fig. 1A-C). The dose kinetics of the ABBOS and Tep69 peptides were strikingly similar (Fig. 1A). In six independent experiments, no difference in dose or time kinetics could be established between Tep69 and ABBOS. On a molar basis, the native proteins, BSA or ICA69 had a 30-50-fold lower activation threshold than the two peptides (p <0.0001, Mann Whitney test), but the two proteins also had near identical dose responses. C3G7 cells and irradiated NOD splenocytes provided necessary and effective APC function, while irradiated C57BL/6 (H-2b) or Balb/c (H-2^d) APC failed to do so (Fig. 1B). These data are consistent with the interpretation of I-A^87 restricted mimicry between ABBOS and Tep69 (129) by a single T cell clone. One testable idea was that the two peptides would have near identical functional properties in vivo.

Alanine scanning of the ABBOS peptide demonstrated that the central residues are critical for T cell responses, including the KAxyKK motif conserved between ABBOS and Tep69 (Fig. 1C). We used peptide response profiles to ALA replacement peptides to estimate the in vivo prevalence of mimicry T cells in NOD mice (Fig. 1D). The bulk proliferative responses of BSA immunized NOD mice resembled the E12.3 profile, since ALA replacement of the identical residues eliminated these responses. The amplitudes of recall responses to BSA, ABBOS, ICA69 and Tep69 were similar; OVA152 peptide served as a negative control in BSA immunized mice. Collectively, these data suggest the presence of E12.3-like, Tep69/ABBOS-specific T cells in NOD mice. Studies measuring the frequency of T cells responsive to both ABBOS and Tep69 is still required for definitive proof.
Figure 1. Cross-reactivity between Tep69 and ABBOS in the E12.3 hybridoma and NOD splenocytes. (A) Antigen dose response curves of E12.3. The T cell hybridoma E12.3 undergoes activation-induced cell death in the presence of C3G7-presented, mimicry peptides or the respective proteins. One of seven experiments with similar results is shown. See Methods for details of the fluorescence-based cell survival assay. The assay was calibrated with untreated, dye labelled E12.3 cells. (B) E12.3 cells are I-Ag7 restricted. I-Ag7-transfected C3G7 lymphoma cells, irradiated spleen cells from NOD, C57BL/6 or Balb/c mice were used as APC's. The same fluorescence-based cell survival assay as in (A) is used. (C) ALA scan of the ABBOS peptide. Peptides with single alanine replacement or (*) replacement of the native alanine residue by Asp (Aspartic acid), were used to stimulate E12.3 hybridoma cells as in (A). Shaded bars: residues conserved in ABBOS and Tep69. (D) Proliferative responses of lymph node cells from BSA immunized NOD mice to the ABBOS-derived ALA replacement peptides were measured by 3H-Thymidine incorporation (mean cpm + 1SD). A limited number of Tep69-derived ALA replacement peptides was tested in parallel. Response profiles resemble that of E12.3 cells (C).
IV.II. Immunotherapy with Tep69 and ABBOS has opposite outcomes

To analyze peptide-based immunotherapy of the Tep69/ABBOS-specific T cells in NOD mice, we employed the adoptive transfer model of NOD diabetes. This model has characteristics of progressive diabetic autoimmunity, and provides a stringent read-out for immunotherapy. Choosing a model with progressive diabetic autoimmunity is important when considering translation to humans with progressive, autoantibody-positive prediabetes (199).

Prior work from Lakshman Gunaratnam showed that engraftment of 0.5, 5, 10, 15, or 20x10⁶ diabetic NOD spleen cells in sublethally irradiated (650 rad) male recipients produced 0%, 20%, 50-60%, 75%, and 100% overt diabetes, respectively, by 35 days post-transfer (200). We aimed for a ~50% incidence to observe both positive and negative peptide effects on diabetes development. Unless indicated otherwise, we transferred 10⁷ diabetic spleen cells in subsequent experiments.

Systemic (i.v.) administration of a single dose of 400 µg of Tep69 peptide one day following spleen cell transfer exacerbated disease development (87% vs 60%, p=0.02, Fig. 2A). Mice injected with OVA152 peptide or PBS developed diabetes at the same rate. In contrast, a single dose of 400 µg of ABBOS peptide one day following transfer resulted in protection from disease (37%, p=0.05). The difference between Tep69 and ABBOS effects were highly significant (p<0.0001). A dose of 100 µg of Tep69 or ABBOS peptide had the same effects as 400 µg (p>0.5, Fig. 2B,C).

Peptide effects could be boosted by repeated administration. Spleen cell donors received 100 µg Tep69 or ABBOS i.v., one day before transfer and the recipient received the same
Figure 2. Tep69 and ABBOS peptides have opposing effects on adoptively transferred IDD. (A). Irradiated NOD males received $10^7$ pooled spleen cells from diabetic donors. The four groups of mice indicated were injected i.v. with a single dose of 400μg of Tep69, OVA152, ABBOS or with PBS only. The graph shows the development of overt IDD in each group. (B). A single peptide injection protocol (100μg) is compared to dual i.v. injection of Tep69 into diabetic donors 1 day before transfer, and into recipients, 1 day after transfer. (C). Single and dual i.v. ABBOS (100μg) injection protocols are compared in mice adoptively transferred with a fully diabetogenic spleen cell dose ($2\times10^7$ cells, i.v.).
treatment one day after transfer (Fig. 2B, C). In such experiments, 100% of mice treated twice with Tep69 developed diabetes. In contrast, dual ABBOS treatment significantly protected from overt disease, even following transfer of larger numbers of diabetic spleen cells (20x10⁶) which caused diabetes in all untreated control mice (Fig. 2C, p=0.0003). Systemic treatment with Tep69 thus exacerbates, while ABBOS prevents disease.

It was unexpected that these two peptides would display opposing in vivo effects, not predicted by their near identical behavior in vitro (e.g. Fig. 1A). We therefore decided to seek confirmation in the other accelerated diabetes model, cyclophosphamide- (CY) induced NOD diabetes (190). Pilot experiments established optimal peptide administration kinetics and CY dose/diabetes relationships, where we chose a relatively low dose that generated overt disease in ~50% of treated mice (250 mg/kg) (200). Intravenous injection of 100 µg Tep69, five days before administration of CY, significantly increased the diabetes incidence 35 days after CY injection (90% vs 40%, p=0.05, Fig. 3A). Animals receiving a low, sub-diabetogenic CY dose (150 mg/kg) developed disease rarely (0-10%), but i.v. Tep69 still precipitated disease (56%, p=0.008, Fig. 3B).

As in the adoptive transfer experiments, i.v. injection of 100 µg ABBOS peptide provided significant protection from CY induced disease (28% vs. 54%, p=0.03, Fig. 3C), with a concordant reduction of insulitis severity (Fig. 4), either counting islets with no or minor islet infiltration (insulitis score ≤1, p=0.0001 vs. PBS treated mice, Fisher’s exact test) or the number of breached islets (insulitis scores ≥2, p=0.0017). This observation maps peptide effects to the islets. Treatments with OVA152 peptide (Fig. 3A, B), or with a Tep69-derived (K(43)=A) alanine replacement peptide (see Fig. 9, below), both resulted in the same diabetes incidences as that in PBS injected mice.
Figure 3. i.v. Tep69 exacerbates and i.v. ABBOS protects from cyclophosphamide-induced IDD. (A) NOD mice received 250 mg/kg cyclophosphamide 5 days after an i.v. injection of 100 μg Tep69 or OVA152 peptide. Animals were monitored for the development of overt IDD until 35 days after cyclophosphamide treatment. (B) The same experiment as (A), except that animals received a subdiabetogenic cyclophosphamide dose of 150 mg/kg. (C) ABBOS (100μg) was injected i.v. instead of Tep69 (CY dose: 250 mg/kg).
Figure 4. High dose i.v. ABBOS reduces insulitis. (A) Islets (100/group) of H&E stained pancreata from i.v. PBS-treated (n=5) or ABBOS-treated mice (n=5). Pancreata were obtained 35 days after cyclophosphamide treatment from mice in either group that had not developed overt IDD. Islets were scored blindly for the presence and severity of islet infiltration using the standard scale (0: no infiltration, 1: peri-insulitis or <25% islet area infiltrate, 2: 25-50% islet area infiltrate, 3: >50% islet area infiltrate, 4: 100% islet area infiltrate or small retracted islet. (B, C) Typical H&E-stained islets from two ABBOS treated, non-diabetic mice at 100x magnification. There is very little insulitis. (D, E) Typical H&E-stained islets from PBS treated, non-diabetic mice at 50x and 100x magnification, respectively. Severe insulitis in many islets.
These data demonstrate that high-dose i.v. administration of Tep69 or ABBOS peptide can exacerbate or protect from disease in two models of progressive NOD prediabetes. The peptide effects were antigen-specific and suggest caution in the design of antigen-specific immunotherapy for the treatment of autoimmune diabetes.

IV.III. Peptide effects require expression of cognate self-antigen

A number of studies associated the outcome of NOD mouse immunotherapies with a shift in overall cytokine bias among autoreactive T cells (186, 201-203), however, the issue is not fully resolved (204). To determine if Tep69 or ABBOS peptides acted through such systemic bystander effects on other autoreactive T cells, we adoptively transferred wild type diabetic NOD spleen cells into NOD congenic ICA69null mice, and treated with i.v. ABBOS or Tep69 as before. Injection of Tep69 or ABBOS in adoptively transferred ICA69null mice had no effect on diabetes development (Fig. 5). Thus, the manifestation of i.v. Tep69/ABBOS effects requires the expression and cognate recognition of the endogenous self-antigen, rather than a prominent systemic bystander effect. Tep69/ABBOS effects thus differ from other immunotherapies, where diabetes development was suggested to be modulated through peptide-induced systemic cytokine release (202) and/or the development of regulatory cells (186). The observation of equal diabetes transfer in PBS-treated ICA69-/- homozygotes and ICA69-/+ heterozygotes indicate, that, like other diabetes autoantigens such as GAD65 (205), ICA69 is a facultative but not obligate autoimmune target in diabetogenesis.
Figure 5. Peptide-mediated modification of adoptively transferred IDD requires expression of the cognate self-antigen. ICA69 deficient and heterozygote male NOD congenics were adoptively transferred with 10⁷ pooled spleen cells from diabetic wild type NOD mice. Groups of 9-11 mice each were i.v. injected as in figure 2, with 100µg Tep69, ABBOS or PBS. The graph shows the incidence of IDD up to 35 days post transfer.
**IV.IV. ABBOS and Tep69 differ in MHC affinity**

The molecular basis for opposite *in vivo* peptide function was unclear, and we asked whether differential MHC class II binding could explain the opposite peptide effects. The relative I-A\(^{87}\) binding affinities of the two peptides were compared by flow cytometry, as well as *in vitro* binding to purified I-A\(^{87}\). Briefly, binding of biotinylated ABBOS to C3G7 cells (67) was easily detected following labelling with FITC-conjugated avidin, while Tep69 binding was weak (Fig. 6A). Tep69 was easily displaced by unconjugated ABBOS, while unconjugated Tep69 displaced very little ABBOS in the dose range tested (Fig. 6B). When Tep69- or ABBOS-loaded C3G7 cells were washed and incubated at 37°C, cell-bound Tep69 was lost rapidly (Fig. 6D). In contrast, ABBOS binding was higher even at one tenth the initial peptide loading dose, and binding was still detectable as late as 24 hours post labelling (Fig. 6C), suggesting an uncommonly high avidity of ABBOS to I-A\(^{87}\). NOD class II may be a poor binder for only some peptides (67). Major differences in I-A\(^{87}\) binding were confirmed with insolubilized, purified I-A\(^{87}\) (Fig. 6E), where only ABBOS binding was sufficiently strong to allow consistent measurement of its binding constant (k=0.344 μM, Fig. 6E, insert); binding of Tep69 was erratic over the dose range tested. Peptide binding to the human PRIESS B cell line homozygous for diabetes-associated class II (197) followed a similar pattern as the C3G7 line, with much lower relative affinity for Tep69 compared to ABBOS (Fig. 6F).

We concluded that ABBOS has a much higher binding affinity to relevant mouse and human class II heterodimers than Tep69 and that, in addition, ABBOS is released from I-A\(^{87}\) at a much slower rate. In functional terms, high on-rates and slow off-rates of ABBOS and the
Figure 6. Tep69 and ABBOS have different affinity for MHC.  

(A) Weak binding of biotinylated Tep69 (thin line) and strong binding of biotinylated ABBOS (solid line) to C3G7 cells. The dashed line are control cells exposed to FITC-Streptavidin only. (B) Competition of unconjugated peptides with biotinylated peptides. Biotin-ABBOS is displaced more readily by ABBOS than Tep69. (C) C3G7 cells were loaded at two loading concentrations and washed. The graph shows the retention of ABBOS peptide over time. (D) An experiment similar to that in (C), except that 200μM Tep69 were loaded. Tep69 peptide is rapidly released from l-Ag7. (E) Binding of biotinylated ABBOS and Tep69 peptides to purified l-Ag7. Insert: determination of the ABBOS binding affinity by Scatchard plot. (F) Weak binding of biotinylated Tep69 (thin line) and strong binding of biotinylated ABBOS (solid line) to human PRIESS cells. The dashed line are control cells exposed to FITC-Streptavidin only.
opposing profile of Tep69 would predict dramatic differences in their in vivo function. On face value, these data clash with the near identity of in vitro peptide dose-responses (Fig. 1A). The two data sets might be reconciled if opposite MHC affinities were balanced by high average T cell receptor affinities for Tep69 (TCR↑) and low averages for ABBOS (TCR↓) (206).

IV.V. Tolerance induction following i.v. peptide treatment

While effector mechanisms of high dose i.v. peptides are generally complex, peptide persistence and sustained TCR stimulation likely contribute to induction of unresponsiveness (180, 207). The assumption of opposite peptide affinity profiles for MHC and TCR’s allowed us to derive some testable predictions. with MHC binding affinity the most important variable in vivo, as it governs the rate of irretrievable peptide loss. In vivo TCR-MHC interactions should 1) be insufficient for induction of long-term unresponsiveness to Tep69, while 2) the opposite would be expected for ABBOS, unless 3) ABBOS doses were sufficiently small to ‘mimic’ the short TCR/MHC interaction time of high-dose Tep69 (206).

To test the first two predictions, 100 μg Tep69, ABBOS, or OVA-152 peptide were injected i.v. as before, followed by immunization with the same peptide in complete Freund’s adjuvant (CFA) four (Fig. 7A-C) or 20 days later (Fig. 7D-F). In vitro proliferative recall responses were measured in lymph node cells obtained 9-10 days after the immunization.

High-dose i.v. injection with any of the peptides generated short term unresponsiveness to the cognate peptide (Fig. 7A-C). PPD control responses were unaffected. As predicted, the unresponsiveness induced in i.v. Tep69 treated NOD mice was transient, and mice immunized 20 days after i.v. Tep69 administration no longer showed unresponsiveness but had elevated
Figure 7. Short and long term unresponsiveness following i.v. peptide treatments. (A-C) NOD mice received a single i.v. injection of 100 μg Tep69, ABBOS or OVA152. Four days later, mice were immunized with the cognate peptide in CFA. Lymph node cells were obtained 9 days later and in vitro recalled with Tep69 (A), OVA152 (B) or ABBOS (C). Proliferative responses to PPD were measured in parallel. All three peptides induce short term unresponsiveness. (D-E) Similar experiment as in (A-C), except that i.v. peptide treated mice were rested for 20 days before immunization and subsequent recall responses to Tep69 (D), OVA152 (E) and ABBOS (F). i.v. ABBOS and OVA152, but not Tep69 induce lasting unresponsiveness.
responses to immunization and in vitro recall (Fig. 7D, F). In contrast, unresponsiveness following i.v. ABBOS (or OVA152) administration was lasting, and immunization 3 weeks after i.v. treatment failed to generate T cell responses as measured by in vitro proliferative recall responses (Fig. 7D-F). ABBOS treated mice also lacked detectable Tep69 responses (Fig. 7D), while i.v. Tep69 treated mice also had high ABBOS responses to immunization three weeks after i.v. injection (Fig. 7F). These data associate long-term unresponsiveness to ABBOS and Tep69 peptides with disease prevention and its failure with disease progression.

The effects of these peptides were further analyzed by measuring in vitro responses in i.v. peptide treated, adoptively transferred and, otherwise unimmunized diabetic mice (the recipients: Fig. 8). Tep69 treated animals not only retained Tep69/ABBOS-specific T cells, but in vitro responses were much higher than those of PBS injected control mice (Fig. 8A, p<0.0001, Mann-Whitney test). Tep69-induced disease exacerbation is most likely due to an expansion of the mimicry T cell pool size. When we analyzed the few (n=3) non-diabetic mice that escaped disease following i.v. Tep69 treatments (Fig. 8B), they were found to lack detectable responsive T cells: only diabetic mice had these cells. In contrast, reactive T cells were undetectable in ABBOS treated mice, including six animals that developed disease despite treatment (Fig. 8A). While ABBOS-induced T cell unresponsiveness is associated with disease protection, this protection appears less absolute than Tep69-mediated disease exacerbation following expansion of this T cell pool.

Our third prediction was that a low i.v. ABBOS dose might mimic the Tep69 effects. This was indeed the case. As shown in figure 9, a fifty-fold decrease in the i.v. ABBOS dose accelerated cyclophosphamide-induced diabetes when compared to controls injected with PBS or the Tep69-derived K(43)⇒ALA replacement peptide. Low dose ABBOS was nearly as effective
Figure 8. High dose i.v. Tep69 expands ABBOS and Tep69 cross-reactive T cells in adoptive transfer recipients. (A) Recipient NOD mice received diabetic adoptive transfer grafts and i.v. Tep69, ABBOS or PBS as before. Proliferative in vitro T cell responses were measured when transfer recipients became diabetic (n=3). Mimicry T cell pools are large in i.v. Tep69 treated mice and absent in ABBOS treated animals. (B) Spleen cells from non-diabetic, and from diabetic, i.v. Tep69 treated mice are compared in their proliferative responses. In mice escaping IDD, i.v. Tep69 treatment failed to expand the mimicry T cell pool.
Figure 9. Low dose i.v. ABBOS exacerbates prediabetes. NOD mice were examined for the incidence of cyclophosphamide induced IDD after receiving a single injection of 3 or 100 µg ABBOS i.v., PBS or 100 µg of the K(43)=>ALA replacement peptide.
as high dose i.v. Tep69 in precipitating disease (p>0.3, Fisher’s exact test).

Collectively, these data suggest that antigens/peptides can have a place in the immunotherapy of established pre-diabetes. Peptides eliciting T cell responses from the same clones do not necessarily have equivalent function and their in vivo action may require cognate recognition of the endogenous self-antigen. Importantly, these data indicate that disease precipitation is a realistic concern in the immunotherapy of progressive diabetic autoimmunity. We discuss, below, how opposing functional profiles of endogenous self- and exogenous mimicry peptides may synergize in the maintenance and progression of diabetic autoimmunity.

V. Discussion

Pre-diabetic autoimmunity targets a fairly constant, similar set of autoantigens in humans and NOD mice (56, 64). Although immunotherapy with any one of these target antigens/peptides can prevent NOD diabetes, effective immunotherapy almost always requires treatment early in prediabetes (56). We consciously focused on models for progressive prediabetes, since human subjects recruited into clinical intervention trials are identified through markers of progressive autoimmunity (199).

The Tep69 effects described here were both, unexpected and unusual, although there is precedence for adverse effects of immunotherapy (204, 208-211). Disease precipitation by Tep69 does not assign a unique, primary role to ICA69 as diabetes autoantigen. Data presented here demonstrate that diabetes develops in ICA69 deficient mice adoptively transferred with wild
type diabetic splenocytes. Like GAD65-deficient animals (205), ICA69null NOD congenics develop IDD spontaneously (Astsatourova, et al., manuscript in preparation), and T cell autoreactivities to GAD65 or ICA69 appear to be facultative rather than obligate or exclusive elements of diabetogenesis.

However, the present data also indicate that in the pre-diabetic islet locale, infiltrated by different pools of autoreactive T cells, the selective expansion of Tep69-specific T cells, as well as their unresponsiveness following ABBOS immunotherapy, can enhance or halt diabetogenesis, respectively. The failure of Tep69/ABBOS treatments in ICA69null NOD mice indicates that systemic bystander effects such as those demonstrated for disease prevention with BCG (202, 212) are not of major relevance here. However, our data do not rule out a possible, peptide-induced cytokine bias within the islet. The failure to observe Tep69 and ABBOS effects in ICA69null mice could then reflect a failure of T cells to migrate to or encounter presented Tep69 in the pancreas. To clarify mechanisms of immunotherapy at cell- and molecular levels we are in the process of generating a mimicry TCR transgene. At this point the most plausible hypothesis is that Tep69 expands the mimicry T cell pool, while high dose ABBOS generates anergic T cells that can migrate to the islet and produce protective cytokines such as IL-4 (213) in response to endogenous ICA69 processed and presented by infiltrating APC. It will be interesting to determine if the same holds true for GAD65-specific autoimmunity, which has been associated with the induction of cytokine bias in some but not other studies (186, 204).

Dramatically different MHC affinities for Tep69 and ABBOS peptides applied to diabetes-relevant human and NOD mouse class II heterodimers. Low MHC affinity of Tep69-like peptides to diabetes-associated DR has been reported by the Roep laboratory (66). To seek and
establish similarities between mouse and human autoimmunity is important when considering translation of murine data to prediabetic humans, and it will be necessary to expand these present studies to the natural disease in NOD females to determine how late in this process peptide therapy is effective, and if repeated administrations are necessary and of benefit.

We propose that the best explanation which reconciles MHC binding data and the near identical peptide dose responses in the E12 hybridoma is opposite affinity profiles for MHC and TCR. Thus, the high MHC affinity of ABBOS would be balanced by low E12 TCR affinity, and the low MHC affinity of Tep69 balanced by high affinity of this TCR for that self-peptide. Thymic ICA69 expression levels are low, but not negative (174). Positive selection would favor high affinity TCRs as few I-A\(^{\gamma}\) molecules would be expected to carry sufficient numbers of Tep69 peptide for periods of time sufficient to stimulate any but high affinity TCRs. Functionally reminiscent of unopposed positive selection (214). there may rarely be enough peptide presentation for negative selection. By themselves, these thymic emigrants are rather unlikely candidates for pathogenic functions, unless these cells are ‘helped along’ by ABBOS in the periphery.

The alternative MHC/TCR affinity profiles would fit with a disease promoting effect of ABBOS, as the small amounts of peptide that may pass enteric censure (215) would be efficiently captured and retained by APCs. We observed that low level ABBOS exposure can enhance disease development and low level natural ABBOS exposure may prevent decline of the mimicry T cell pool due to neglect. However, once the islet is breached and invasive insulitis established, more endogenous Tep69 becomes available to lesional APCs, sufficient for activation of the mimicry pool by its cognate self-antigen. In this sense, protection and exacerbation may be viewed as a numeric variable that describes the size of the Tep69/ABBOS-
controlled mimicry T cell pool and its dynamic change through peptide exposure. Figure 11 summarizes a model depicting the role of ABBOS and Tep69 in diabetes development.

Antigenic mimicry is a prominent concept in the pathoetiology of autoimmune diseases, but examples of naturally occurring mimicry are infrequent (216, 217) and mechanisms that lead to disease are not always clear (30). The implicit assumption of equivalent functions is common, and probably at times correct, but is rarely tested.

ABBOS-containing BSA fragments are naturally generated following ingestion of cow milk (215). Complex, multi-antigenic weaning diets are a prerequisite for diabetes development in NOD mice and BB rats, and these rodents are solidly protected from disease when weaned to non-antigenic diets such as casein hydrolysates (1). Hydrolyzed diets prevent the development of Tep69/ABBOS mimicry T cells, and consistent with a role of diet in the shaping of diabetogenic T cell repertoires, such mice do not respond to immunization with ICA69, even in complete adjuvant (164). However, the role of cow milk proteins is controversial (167), and addition of BSA in drinking water of hydrolysate-fed BB rats did not lead to diabetes, but instead afforded a small, but significant protective effect (168): in this report, consumption of several milligrams of daily BSA in mothers and offspring may have provided sufficient systemic ABBOS exposure for protective ABBOS effects early in the life of some animals.

Collectively, our observations have uncovered several unexpected facts, and suggest that immunotherapeutic targeting of a common mimicry T cell pool with high affinity ABBOS, and possibly homologs with higher MHC affinity, can halt progression of diabetic autoimmunity. Opposing effects of mimicry peptides provide a plausible scenario of how these naturally generated pair of mimics may act in a synergistic fashion. Environmentally acquired ABBOS
Figure 10. Proposed role of mimicry peptides in diabetes development.
Thymic epithelium expresses low levels of the low MHC affinity Tep69 peptide. Positive selection of mimicry cells would therefore favour high affinity TCRs. After exposure to dietary BSA, low quantities of ABBOS are found in the gut. Since low doses of ABBOS are diabetogenic, the low quantities of dietary ABBOS can maintain the mimicry cell pool at significant levels. After establishment of insulitis, and subsequent beta cell destruction, large quantities of ICA69/Tep69 are liberated from dying beta cells and presented by antigen presenting cells, likely in the islet or pancreatic lymph node. The high concentration of local Tep69 can fully activate mimicry T cell effector function and contribute to disease.
would sustain thymic emigrant mimicry cells, while Tep69-mediated effector function in the islet would be enhanced when self-antigen supply from dying β-cells reaches significant levels in progressive prediabetes. Our data suggests caution in the design of immunotherapies applied during mid- to late-stage prediabetes, where T cell access to the target organ has already appeared. The similarities of mimicry T cell pools and ABBOS and Tep69 affinities in humans and NOD mice are striking, making this an attractive system for the development of a tailored immuno-therapeutic strategy. The peptide effects were clearly boostable, and repeated peptide treatments can be considered when the reappearance of target T cells is detected. Peptides that have homologues in relevant animal models, parallel study of humans and rodents, peptide dose, and MHC affinity should be factors in the design of immunotherapeutic peptides considered for human use.

VI. Future Studies/Synopsis

This project has created several opportunities for further investigations. One major implication of the study is that in NOD mice, higher MHC-affinity peptides are superior immunotherapeutic agents compared to lower MHC-affinity peptides (in the correct dose). Thus, it seems logical to generate an ABBOS-analog peptide with considerably higher affinity. The peptide would be constructed by measuring relative affinities of alanine replacement ABBOS analogues (without replacing any critical residues) and compare them with wild type ABBOS. The NOD mouse provides an ideal model to develop and test high-affinity ABBOS analogues that might be considered for human intervention trials, based on the closely analogous binding characteristics of human diabetes-associated DR/DQ molecules. The critical test will be to
determine if high affinity ABBOS analogues are indeed more efficient immunotherapeutic agents than wild type ABBOS.

A greatly enhanced affinity ABBOS-analogue should minimize potential harmful effects from inadvertent under-dosing in humans, since it would have a wider therapeutic range and a lower dose of the analogue would still be protective *in vivo*. We are now able to identify high risk (70% chance of diabetes in 15 years) first-degree relatives on the basis of MHC and autoantibody risk markers. A high affinity ABBOS-analogue could be an attractive peptide candidate for human intervention trials.

There are additional experiments which could be performed to confirm and extend data in this thesis. Probably the most important experiment would be to test ABBOS immunotherapy in 10-12 and 16-17 week old NOD mice, and measure the peptide effects on the spontaneous development of diabetes. This will be a major experiment that requires large animal numbers and prolonged observation periods, since it is likely that several treatment regimens with different doses and repeated administrations will need to be evaluated through disease outcomes as well as proliferative T cell assays.

It would be interesting to determine which peptide is dominant in a high dose of ABBOS and Tep69 co-injection. Data from the thesis would predict ABBOS to be dominant due to its higher MHC affinity and lower off-rates. Similarly, it would be reasonable to determine if injection of 3 µg of ABBOS peptide induces transient tolerance or hyper-proliferation similar to the injection of 100-400 µg of Tep69. Data presented in the thesis would predict transient tolerance and hyper-proliferation, since 3 µg of ABBOS peptide precipitates diabetes much like 100-400 µg of Tep69. Other intriguing questions include determining whether a prolonged
exposure to Tep69 would induce tolerance and prevent disease, and determining the in vivo peptide dose at which ABBOS "switches" from disease protection to disease exacerbation.

It would also be attractive to determine possible cytokine bias (Th1 versus Th2) in the islet locale. It would be predicted that intravenous ABBOS induces a Th2 bias in the islet of NOD mice, but fails to do so in the islets of ICA69null NOD congenics. Quantitative RT-PCR from islets isolated from collagenase digested pancreata was attempted, but results were unreliable due to poor RNA quality, possibly caused by a poor islet isolation technique.

Finally, considerable effort should be allocated to creating an ABBOS/Tep69-specific TCR transgenic NOD mouse. With this mouse, it would be possible to further elucidate the underlying mechanism behind the effects of ABBOS and Tep69 treatment, since we would be able to follow the behavior of antigen specific T cells. Does high dose ABBOS induce mimicry, T cell anergy, apoptosis, altered signaling? An ABBOS and Tep69-specific TCR transgenic NOD mouse would provide us with a tool to address such questions.

VII. References


Diabetes 33: 271.


Diabet Med 7: 731.


DiLorenzo, T. P., R. T. Graser, T. Ono, G. J. Christianson, H. D. Chapman, D. C.
class I-restricted T cells are required for all but the end stages of diabetes development in
nonobese diabetic mice and use a prevalent T cell receptor α chain gene rearrangement.
Proc Natl Acad Sci USA 95: 12538.

Serreze, D. V., H. D. Chapman, D. S. Varnum, M. S. Hanson, P. C. Reifsnnyder, S. D.
the initiation of T cell-mediated autoimmune diabetes: analysis of a new "speed congenic"

B-cells are required for the initiation of insulitis and sialitis in nonobese diabetic mice.
Diabetes 46: 941.

Med 189: 347.

requirement of macrophages for the development and activation of beta-cell cytotoxic CD8+
T-cells in T-cell receptor transgenic NOD mice. Diabetes 48: 34.

Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese


(133) Sarvetnick, N., J. Shizuru, D. Liggitt, L. Martin, B. McIntyre, A. Gregory, T. Parslow and


Immunogenetic determinants and prediction of IDDM in French schoolchildren. *Diabetes* 44: 1029.


Pediatrics 94: 752.


