

GAD IN METABOLIC & NEUROLOGIC DISEASE

DMCCAD, STOCKHOLM, SWEDEN, JUNE 2003

PARKINSON'S
LADA
HUNTINGTON'S
BATTEN'S
DIABETES
OBESITY
SMS

GAD
10-65702-13-01
1mg/ml GAD65

T CELL GAD
10-65702-13-01
1mg/ml GAD65

T CELL GAD
10-65702-13-01
1mg/ml GAD65

rhGAD65
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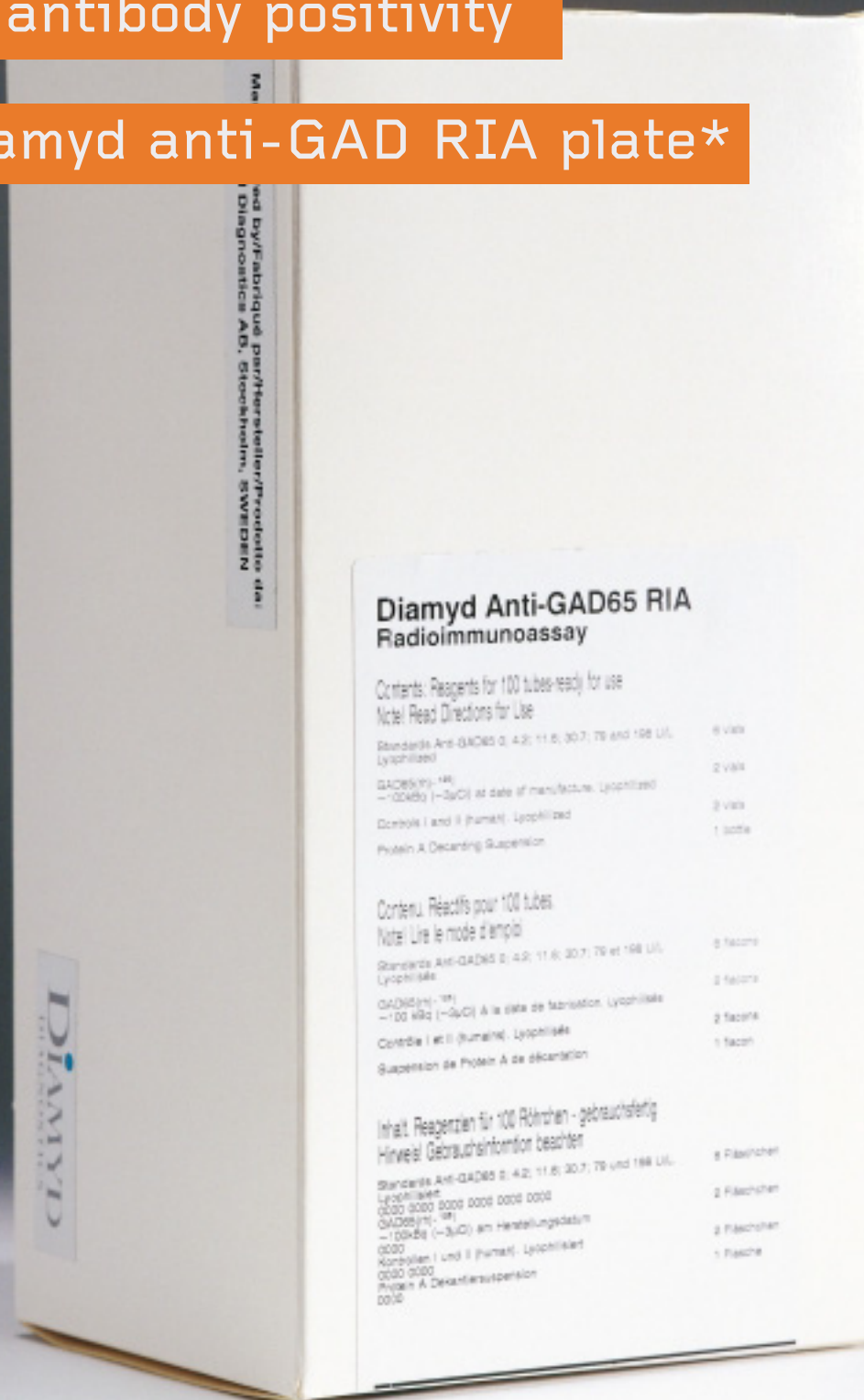
10 % of your "Type 2" diabetes patients

may in fact have LADA (Latent Autoimmune Diabetes in the Adult)

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with the Diamyd anti-GAD RIA plate*

*US patent # 5547847
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Marcovina *et al*,
Evaluation of a novel radioimmunoassay using ¹²⁵I-labelled human recombinant
GAD65 for the determination of glutamic acid decarboxylase (GAD65)
autoantibodies
Int J Lab Res (2000) 30: 21-26

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DMCCAD

In April 1999 DIAMYD MEDICAL initiated a new Competence Group for Autoimmune Diabetes (DMCCAD) at the Centre of Molecular Medicine (CMM) at the Karolinska Hospital. The Company appointed *Associate Professor Robert Harris* to lead this research group.

FOREWORD

Research Scientists throughout the world are currently faced with a time-old challenge – to define and understand the mechanisms leading to development of autoimmune diseases, and then to determine and develop efficient means of treating or preventing them. While these might sound like two distinct challenges, the definition of the molecules targeted in an autoimmune disease process also provides the candidates for therapeutic targeting.

To date, there is no vaccine for any of the many autoimmune diseases that affect millions of people throughout the world. A number of candidate molecules have been identified and targeted, such as insulin in Type 1 diabetes and myelin basic protein in Multiple Sclerosis, but clinical vaccination trials using these molecules have not yet been successful. Given the recent advances in gene technology and knowledge of the genetic codes comprising both Man and experimental animals, the potential for discovery of new candidate molecules is great. However, one candidate molecule identified many years ago still stands the test of time.

Glutamic acid decarboxylase 65 (GAD65) is a candidate autoantigen implicated in development of the autoimmune disease Type 1 diabetes. In autumn 1994, 'The Story of GAD' by Robert Dinsmoor appeared in the Countdown magazine, a publication of the Juvenile Diabetes Research Foundation International. This article described the series of research discoveries encompassing the period 1982-1993 that led to definition of GAD65 as a prime candidate autoantigen in Type 1 diabetes. The article ended with two pertinent statements – that access to recombinant GAD in industrial-sized quantities would be required for testing of GAD therapies in humans, and that GAD-based therapies should provide a means of preventing diabetes.

During the last 10 years, intensive efforts have led to development of high quality recombinant GAD65 proteins, which have been tested in Phase I and Phase II clinical trials.

Additionally, recent research efforts have also identified GAD as an important element in Parkinson's and other neurological diseases, indicating that the candidate molecule has not only remained a potential key to prevention of diabetes, but also important in treatment of other diseases as well. There is obviously still more to learn about this fascinating protein.

This issue is dedicated to GAD65 with a number of contributions made by several prominent GAD researchers. As well as personal reflections of their own past experiences of GAD, the current status and proposals for future applications of GAD are presented. We hope you find these articles interesting.

Assoc. Prof. Robert A. Harris



INTRODUCTION

Introduction by Lars Klareskog

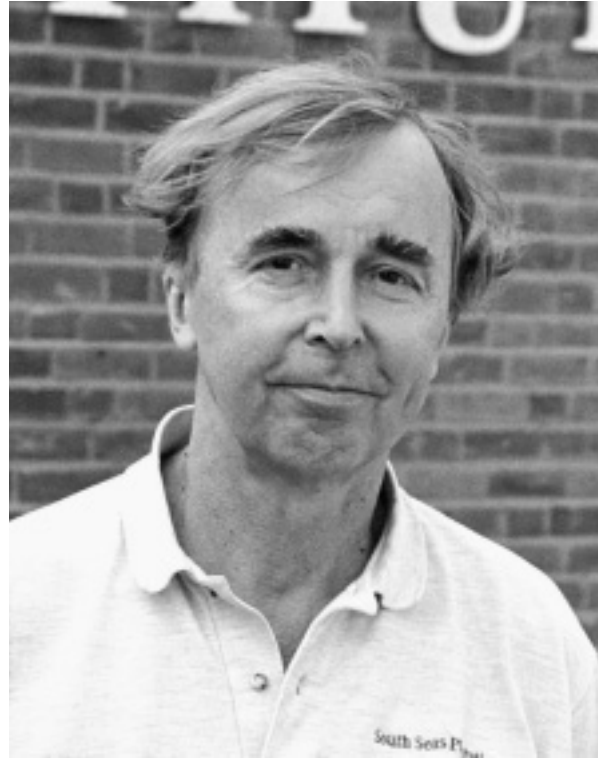
In 1999 DIAMYD MEDICAL initiated a unique cooperation with the Karolinska Hospital when setting up its Competence Center for Autoimmune Diabetes (DMCCAD) at the Center for Molecular Medicine (CMM) at Karolinska. Since its inception, with Robert Harris at the helm, DMCCAD has become an interesting point for cross-fertilization of ideas related to various autoimmune diseases including Type 1 diabetes, MS and Rheumatoid Arthritis.

It is generally agreed that Glutamic Acid Decarboxylase 65 (GAD) is an important antigen in Type 1 diabetes. As in other autoimmune diseases, recent research is focused on induction of tolerance to disease specific antigens. This may protect self structures under attack without interfering with the immune system's capabilities in other areas, such as combatting viral infections or cancer.

While it is already generally accepted that GAD antibodies (GADA) is a major diagnostic marker for Type 1 diabetes and predicting the course of development of Type 2 diabetes (LADA-patients), tolerance induction to GAD may become a way to prevent the development of insulin dependency in LADA patients. I have had the pleasure of being able to follow the development of Diamyd Medical's GAD-based vaccine for LADA patients. The outcome of the Phase II study in LADA patients is important, not only because it may lead to a new drug for diabetes therapy, but its results may also be of importance when designing future clinical trials in other diseases such as MS.



Lars Klareskog, MD, Ph.D., is Professor of Rheumatology and Head of the Rheumatology Research group at the Karolinska Hospital in Stockholm and formerly Professor of Medical Immunology and Head of Clinical Immunology at the Uppsala Teaching Hospital. Klareskog's research is specifically aimed at the causes and treatment of autoimmune disorders. Klareskog is a member of the Nobel Foundation.



Hans Wigzell, MD, D.Sc. is Professor of Immunology, Dean of the Karolinska Institute and Chief Scientific Advisor to the Swedish Government. Wigzell is one of Sweden's most prominent and internationally renowned scientists in the field of Immunology. Wigzell was Director General of the National Bacteriological Laboratory 1988-1993; Director General of the Swedish Institute for Infectious Diseases 1993-1994; Wigzell is since 1990 Chairman of the EC Concerted Research Programme: European Vaccine against AIDS (EVA). Wigzell was Chairman of the Nobel Committee 1990-1992 and is Chairman of the Nobel Foundation.

Comments on *Diamyd Diabetes Vaccine* by Hans Wigzell

That the human immune system can react against self structures is well known. Most such reactions do not cause disease – but some do.

In the efforts to fight autoimmune disease such as Type 1 diabetes, one therapeutic approach is to modify the immune reaction and induce functional tolerance to potentially relevant molecules such as GAD by administration of the autologous antigen itself.

Similar approaches have been successful before. For example administration of autoantigens have alleviated autoimmune disease in animal models. Hyposensitization (where increasing doses of allergen are used to treat allergies) is another example.

How should GAD be used to induce functional tolerance? Using our knowledge from conventional as well as from autologous vaccines it seems logical to use doses from a few micrograms to 500

micrograms.

To use alum as adjuvant seems highly recommendable. It is conventional and it is biased to the humoral rather than the cellular immune response which is logical when the cellular response is to be reduced.

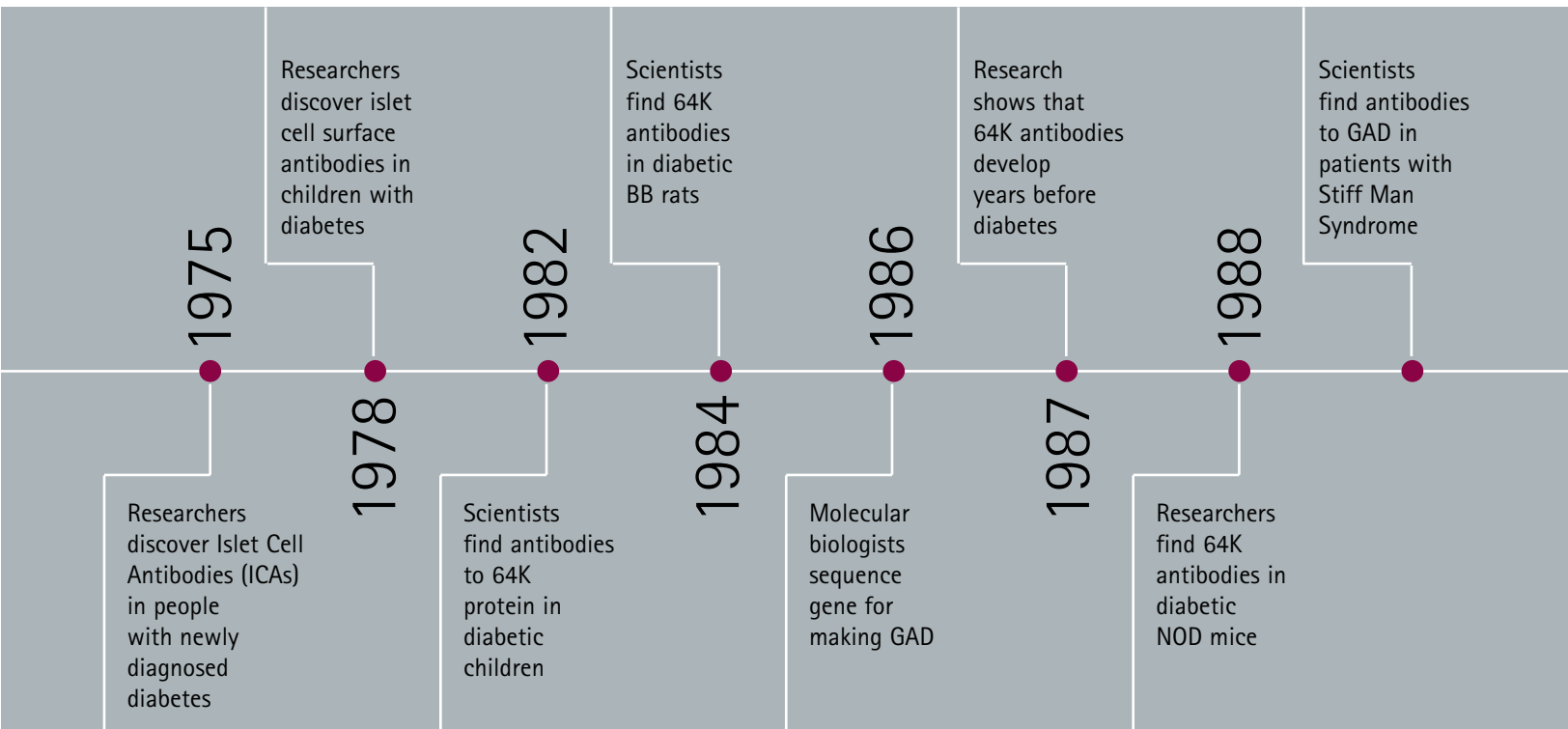
Subcutaneous administration is recommendable and conventional. Intravenous administration is much more problematic and intramuscular is less efficient in terms of immunogenicity.

The GAD-vaccine differs from a conventional vaccine in that the administered antigen is already present naturally in the body. This probably means that the vaccine needs to be injected a couple of times. Therefore a prime and boost strategy seems logical.

In summary, the DIAMYD approach to induce functional tolerance to GAD seems logical.

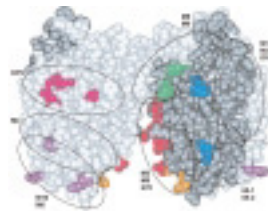
The Story of GAD

Robert Dinsmoor



This article's original form appeared in Countdown magazine, a publication of the Juvenile Diabetes Research Foundation International, Autumn 1994.

At one time, scientists thought that diabetes was caused by a virus or toxic environmental agent that destroyed the insulin-producing beta cells of the pancreas. But decades ago, scientists discovered that people with newly diagnosed diabetes had inflammation of the islet cells of the pancreas, or insulinitis.

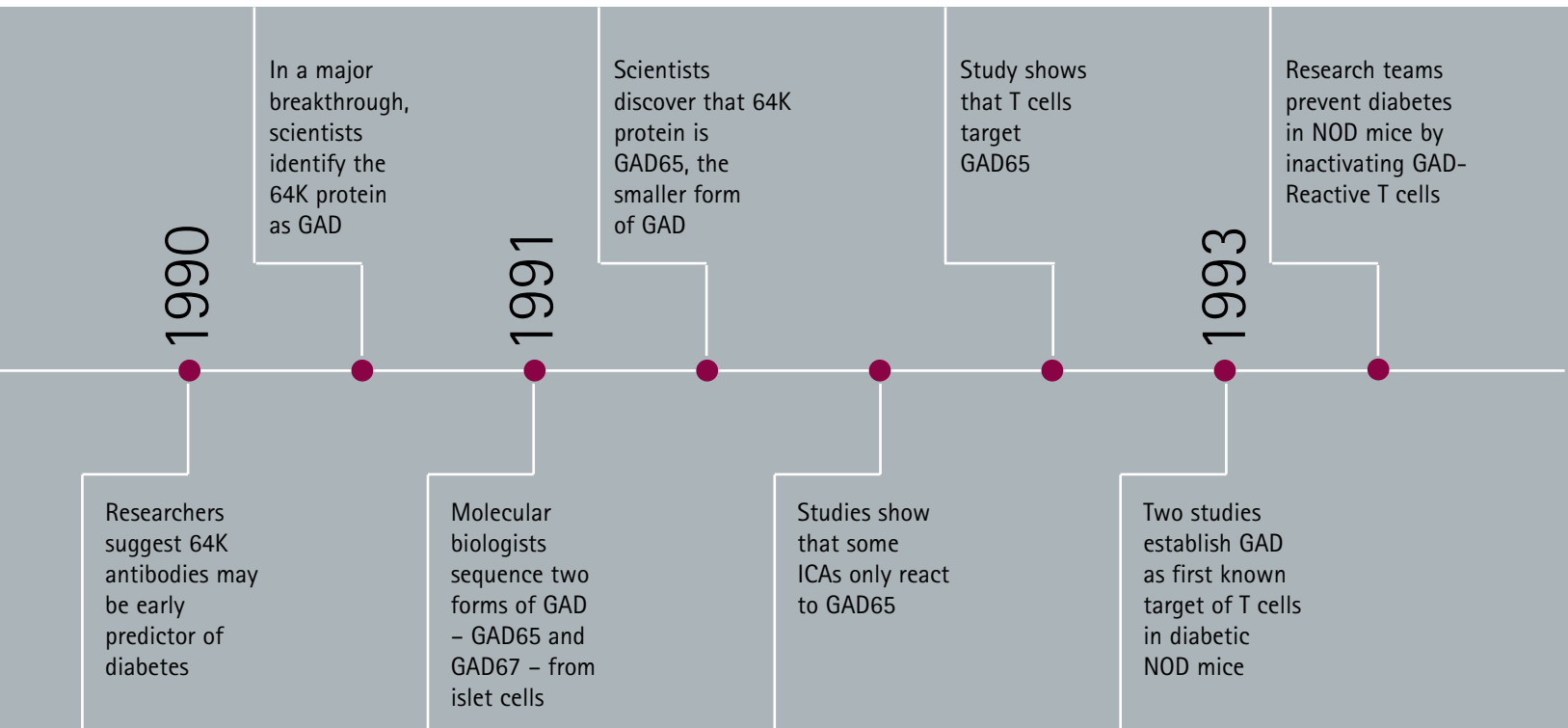


The islets were filled with white blood cells, which the body's immune system uses to fight infection. This led researchers to speculate that diabetes might be an autoimmune disease, caused by a misdirected attack by the body's own immune system.

More support for the autoimmune theory came in the early 1970's when *Dr. Bottazzo* and *Dr. Doniach* and colleagues at the Middlesex Hospital in London first detected antibodies to pancreatic islet cells in people with newly diagnosed diabetes. The antibodies, which react to the cytoplasm inside all islet cells, came to be called cytoplasmic islet cell antibodies or ICAs.

Antibodies are a part of the immune system response called the humoral response. Made by white blood cells called B lymphocytes, specific antibodies are designed to attack specific foreign proteins or antigens. It was unclear, however, whether the beta cells were actually destroyed by these antibodies or by components of the cellular immune response, such as the killer T lymphocytes or "T cells".

In the mid-1970's, *Dr. Lernmark*, who had developed a technique for isolating beta cells from isolated islets of

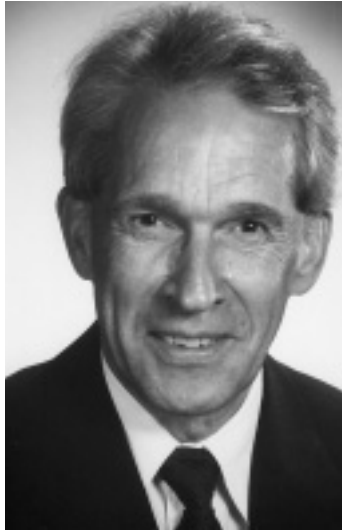


the Langerhans, was researching proteins on the surface of beta cells at the University of Chicago. While involved in this research, he attended a lecture in ICAs in Chicago by *Dr. Doniach*. During the lecture, he realized that it must be the proteins on the surface of the beta cells that provoked antibodies in diabetes, and he turned his research in that direction. He and other researchers at the University of Chicago treated pancreatic islets cells from rats with blood serum from children who had diabetes for less than five years. They discovered that some of these children

had antibodies to proteins on the surface of the islet cells, called islet cell surface antibodies or ICAs. However, they did not know which specific proteins had attracted the antibodies.

The 64,000 Mystery Protein

In the late 1970's, *Dr. Lernmark* became Director of Research at the Hagedorn Research Laboratory in Denmark, where he continued to work with ICAs. He enlisted the aid of *Dr. Baekkeskov*, who had developed



Åke Lernmark, MD, Ph.D., and his colleagues at Hagedorn Research Laboratory in Denmark, first reported 64K proteins in humans in 1982.



Steinunn Baekkeskov, Ph.D., played a primary role in detecting the 64K protein in humans and later worked in collaboration with Yale researchers to identify the protein as GAD.



Mark Atkinson, Ph.D., and colleagues at the University of Florida in Gainesville, concluded in 1990 that 64K autoantibodies might be the earliest and best marker for diabetes yet identified.

techniques for detecting membrane proteins while studying the role of antigens in African sleeping sickness. Using newly developed and very sensitive tests such as the “immunoprecipitation technique,” Drs. Baekkeskov, Lernmark and colleagues at the Hagedorn found 80-90 percent of blood serum samples from children with newly diagnosed diabetes had antibodies to an unidentified ICSPA with molecular weight of approximately 64,000 daltons or 64 kilodaltons. They dubbed the mystery protein 64K, and reported their findings in 1982 in *Nature*.

In 1984, they reported that 64K antibodies were often found in diabetic BB rats (a commonly used animal model for human diabetes). One intriguing aspect of this finding was that the antibodies appeared 40 to 70 days before the onset of diabetes. This suggested that the 64K protein was an early and major target of the immune system. But did this same predictive effect hold true for humans as well?

They next looked at blood serum samples of 14 people who later developed diabetes. Eleven of them had antibodies to the 64K protein - for up to seven years before they began to show symptoms of diabetes. This suggested that 64K antibodies might be an early warning sign for the eventual development of diabetes.

In the summer of 1986 *Dr. Atkinson* who had been studying environmental influences on diabetes in rats at the University of Florida in Gainesville, traveled to Hagedorn as a visiting scientist. There Baekkeskov taught him the immunoprecipitation technique for detecting 64K antibodies. Armed with this new skill, he returned to the University of Florida, where he and *Dr. Noel*

Maclaren, detected the 64K antibodies in nonobese diabetic (NOD) mice, a newly bred type of animal model for diabetes. Finding the 64K antibodies in animals helped confirm that the 64K antigen was an important feature in diabetes.

Drs. Atkinson, Maclaren, and colleagues also studied first-degree relatives of people with diabetes, who were at higher risk for developing diabetes themselves. In 1990, in the British Journal *The Lancet*, they reported finding 64K autoantibodies in 23 of 28 people who developed diabetes up to seven years later. Based on these findings and those of the Hagedorn researchers they concluded that 64K might be the earliest and best marker for diabetes yet identified and might be especially useful for predicting diabetes.

As diabetes researchers became increasingly aware of how important this protein was, they set out to analyze its chemical characteristics to compare with known proteins.

In 1988, *Drs. Solimena, Camilli*, and their colleagues at Yale University reported in *The New England Journal of Medicine* that they had found the target antigen in patients with an autoimmune disorder called Stiff-Man Syndrome (“SMS”). SMS is a rare disorder of the central nervous system in which a person’s muscles become more and more rigid, and painful spasms occur. Patients with the disorder were found to have antibodies to an enzyme called Glutamic acid Decarboxylase, or GAD.

GAD is responsible for converting the amino acid glutamate into a protein called GABA, which the brain cells use to communicate. It turned out that many patients with

GAD antibodies had diabetes – even the ones who never developed SMS. Moreover, they found that antibodies from the serum of SMS patients targeted the beta cells, and these were not any of the previously known antibodies.

This led to an historic collaboration between Dr. Baekkeskov, now at the University of California, San Francisco, and the Yale research team. Dr. Camilli sent Baekkeskov blood serum samples from SMS patients (containing GAD antibodies), and she sent him blood serum samples from diabetes patients (containing 64K antibodies). Then they used various methods to demonstrate that the proteins were completely identical. The end result of this meeting of the minds was their landmark paper in *Nature* identifying the 64K protein as the enzyme GAD. The discovery set off a flurry of interest in GAD by diabetes researchers.

Send in the Clones

Dr. Kaufman, who had been studying the role of GAD in neurological diseases such as epilepsy, became interested in diabetes one day in 1990 when his car broke down. While waiting for it to be fixed, he went to the library, where he stumbled on the article by Dr. Atkinson and colleagues in *The Lancet* describing the 64K antibodies as predictors of diabetes.

According to Kaufman, the landmark paper identifying 64K as GAD had yet to appear in *Nature*, but he was able to put the two together for himself: He knew about the role of GAD in SMS and the fact that it was found in beta cells, and he also knew that that molecular weight of GAD was approximately 64kD. He joined forces with the research team at the University of Florida, as well as with his former mentor at the UCLA *Dr. Tobin*, who was researching the role of GAD in Huntington's disease and epilepsy. There were known to be slightly different molecules of GAD found in the brain – one weighing 67kD (GAD67) and a smaller one weighing 65kD (GAD65). As a graduate student working with Tobin, Kaufman had worked out the genetic sequence for GAD67, and another graduate student *Dr. Erlander*, had done the same for GAD65. Thanks in part to this work, Dr. Tobin's laboratory was now able to make both forms of GAD in quantity using genetically altered bacteria.

Spurred by the news that 64K was GAD, other research groups cloned GAD. In 1988, Dr. Lernmark had moved to the University of Washington in Seattle with some members of his former Hagedorn research team. One of these *Dr. Allan Karlsen*, cloned the gene for human islet GAD65. One of Lernmark's former graduate student's, *Dr. Birgitte Michelsen*, cloned GAD67. According to Dr. Lernmark, cloning GAD enabled researchers to make recombinant GAD in quantities they had never dreamed



Allan Tobin, Ph.D., and Daniel L. Kaufman, Ph.D., at the University of California at Los Angeles published one of two 1993 papers in "Nature" confirming that GAD is the first known target of the T cell attack in diabetes.

of. Cloning GAD65 also enabled researchers to determine which molecule was the alter ego of 64K.

The researchers in Tobin's lab sought help from a UCLA diabetes expert, *Dr. Michael Clare-Salzler*, to study GAD's role in diabetes. Using blood serum samples from diabetic patients with newly diagnosed diabetes provided by Dr. Clare-Salzler, the researchers showed that 96% of patients with newly diagnosed diabetes had antibodies to one or both forms of GAD. Furthermore, it appeared that 64K was GAD65, the smaller of the two molecules that had been identified.

Dr. Erlander also used a computer search that uncovered structural similarities between both forms of GAD and proteins in the Coxsackie virus, a virus known to infect many people who eventually developed diabetes. "That moment was probably the most exciting moment in my whole scientific life!" Dr. Tobin recalled. This finding supported a theory known as "molecular mimicry", which suggested that infection by the Coxsackie virus might set off the immune reaction in diabetes. According to the

“ As diabetes researchers became increasingly aware of how important this mystery protein was, they were eager to discover its true identity ”



Hugh McDevitt, at Stanford University suggested in 1993 that diabetes could be prevented in its earliest stages by preventing the T cell attack against GAD.

theory, the immune system mistakenly attacks GAD in the beta cells as well.

GAD Provokes an Immune Attack

In 1992, a number of studies showed that some of the ICAs, the antibodies described back in 1974 that were very predictive for diabetes, targeted GAD. One study from Bottazzo's research group in London found that ICAs from the blood serum samples of pre-diabetic individuals seemed to attack GAD. Likewise *Dr. George Eisenbarth* and *Dr. Robert Gianini* and colleagues at Joslin Diabetes Center in Boston studied ICAs from relatives of people with diabetes. They found that GAD was one target of ICAs, but there might be other antigens as well.

These reports showed an attack on GAD by the humoral immune system – that is antibodies. But these antibodies are probably not what destroys the beta cells. More likely, they are destroyed by the cellular immune response in the form of T cells, white blood cells that infiltrate the pancreatic islets.

The same year, Drs. Atkinson and Maclaren, working in conjunction with the UCLA research team, took T cells from people with newly diagnosed diabetes, relatives of people with diabetes, and nondiabetic individuals, and exposed them all to GAD65. The T cells that reacted and multiplied in the presence of GAD65 tended to be those from people with diabetes and their relatives who had ICAs and would later develop diabetes. This showed that GAD provokes a T cell attack, which may be what destroys the beta cell in diabetes.

In 1993, two studies published in *Nature* helped confirm GAD as the first known target of the T cells and suggested that diabetes could be prevented in its earliest stages by preventing the T cell attack against GAD. One study was from Kaufman and colleagues at UCLA in conjunction with Dr. Atkinson, and the other was from *Dr. Hugh McDevitt*, *Dr. Roland Tisch* and their colleagues at Stanford University. In each case, the researchers tested NOD mice for reactivity to some known antigens in diabetes, including the two forms of GAD. They found that the attack on GAD by T cells coincided with the development of insulinitis – infiltration of the beta cells with white blood cells. Only later did the T cells attack other known antigens in the beta cells.

This suggested that GAD is the first antigen to be attacked by T cells, and that this attack then diversifies to

include other antigens, and that these were recognized and attacked by the immune system. Was this actually the cause, or was there some other antigen, as yet unknown, that provoked the first attack?

To help answer these questions, both groups of researchers tried deactivating the T cell response in their NOD mice. The UCLA group injected NOD mice with GAD at three weeks of age, a treatment already shown to inactivate T lymphocytes against GAD. Most of the mice treated with GAD showed no T cell response against GAD, indicating complete immune system tolerance to GAD. The GAD tolerant mice showed no reaction to the other beta cell antigens, and never developed any degree of insulinitis or diabetes. This suggested that GAD – and not any of the other known antigens – provoked diabetes. On the other hand, mice treated with other antigens did develop a T cell attack to the other beta cell to antigens, did develop insulinitis, and went on to develop diabetes.

The Stanford researchers likewise tried inactivating GAD-reactive T cells – but in this case by injecting NOD mice with GAD65 into their thymus (a major site of immune system programming). The 70 percent of NOD mice who actually became tolerant of GAD had markedly reduced T lymphocyte responses to the other beta cell antigens, reduced insulinitis, and no development of diabetes.

"I think this is an important step toward understanding the disease process," Dr. Tisch explained. "It appears that the disease occurs in specific stages, and now we may be able to categorize the antigens with regard to reactivity within those stages."

The Prediction and Prevention Pay-Off

Now that GAD has been identified, cloned, and shown to be important in the development of diabetes, it can play an important role in diabetes treatment, to predict who will get diabetes – probably years before they develop any symptoms of the disease. Now we have a very specific assay to show whether a healthy person has GAD antibody. But the ultimate implications of GAD go far beyond predicting who will get diabetes. The recent studies in NOD mice show that GAD, too, might play a role in preventing diabetes.

Unfortunately, testing oral GAD in humans won't be feasible until researchers have access to recombinant GAD in industrial-sized quantities and at an affordable cost. "We've actually discovered a direction for potentially preventing diabetes in human beings. Using treatments such as with GAD we should be able to prevent diabetes in all individuals who are at risk of developing it," concluded Dr. Clare-Salzler.

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GAD Back to the Future...

Åke Lernmark



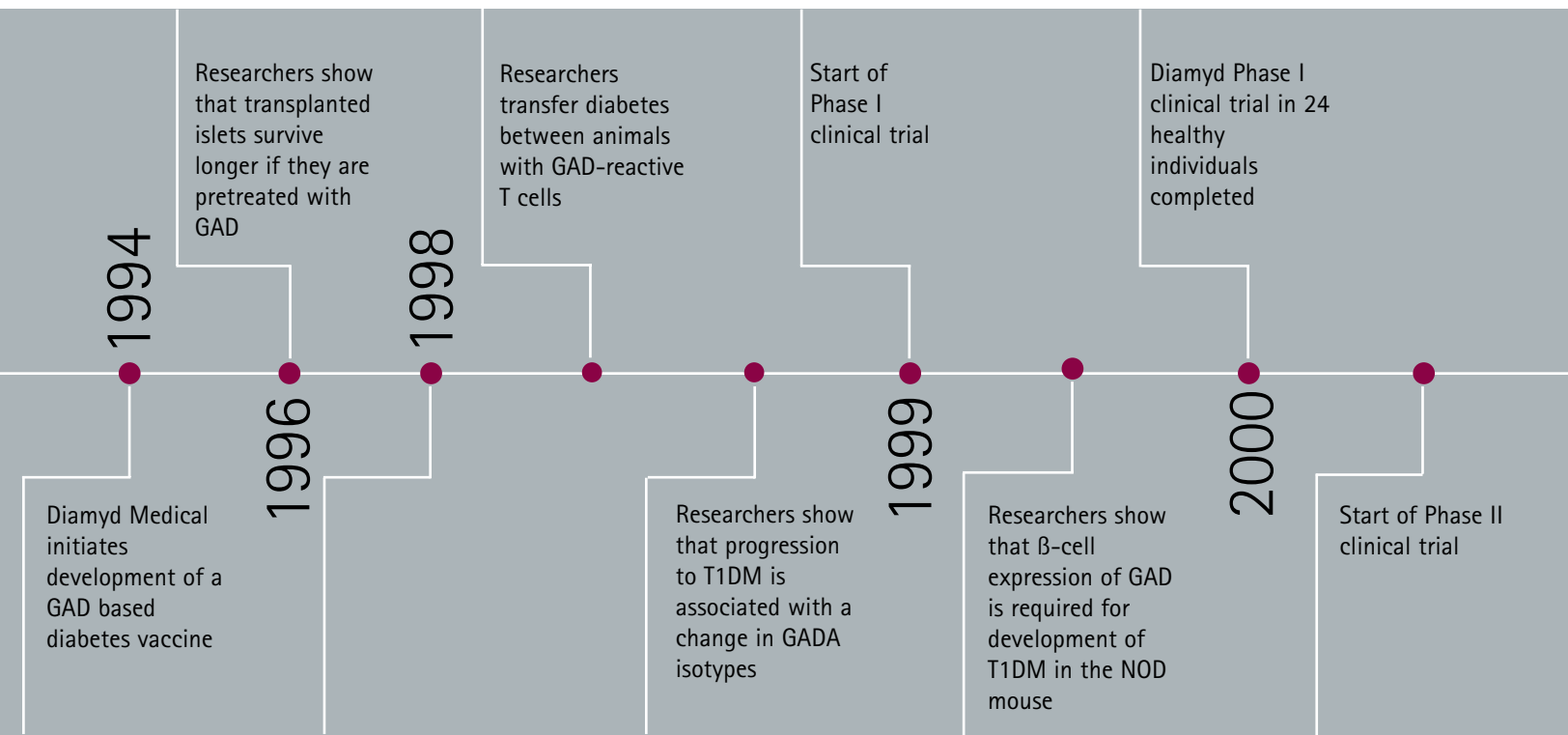
Åke Lernmark, MD, Ph.D., is R.H. Williams Professor of Medicine at the University of Washington in Seattle. Lernmark focused his attention on diabetes and the antigen that later proved to be GAD at an early stage. Lernmark was first to demonstrate the presence of antibodies against GAD in patients with insulin-dependent diabetes, and to use molecular methods to define HLA genes that are necessary, but not sufficient to develop the disorder.

The beta cell specific loss associated with the clinical onset of Type 1 diabetes mellitus (T1DM) is a remarkable phenomenon. The ablation of the insulin-producing cells does not seem to affect neighboring cells producing glucagon, somatostatin or pancreas polypeptide. The eradication of the beta cells is a testimony to the laser knife-like precision of the immune system to recognize and attack antigen. The immune mediated loss of beta cells is well documented in pancreas specimens from T1DM patients of short disease duration. The degree and character of the inflammatory cell infiltration vary from patient to patient and would not predict clinical onset. The loss of beta cells appears more predictive and the major question that we and others try to answer is whether the immune attack on the beta cells can be reversed by controlling the autoimmune reaction against glutamic acid decarboxylase (GAD65) either before or after onset. While autoantibodies to insulin and IA-2 are predictive of T1DM primarily in the young, the predictive value of GAD65 autoantibodies appears less age-dependent. It is therefore often argued that GAD65 is the major autoantigen in T1DM, which is consistent with GAD65 autoantibodies being most prevalent (more than 80% of new onset patients) at onset. Although recent follow up studies suggest that GAD65 autoantibodies are not necessarily the first autoantibody to appear before the clinical diagnosis is made, appearance of all three autoantibodies to GAD65, IA-2 and

insulin is the best predictor of disease. Recognition of GAD65 by helper CD4 and killer CD8 positive cells are likely to precede the autoantibody appearance. At present, there is a lack of reliable and standardized tests that detect T cell reactivity against islet autoantigens (1). This is in contrast to GAD65 and IA-2 autoantibodies, which can be reliably measured against a WHO standard (2). At least there will be one reliable way to monitor whether Specific Immune Therapy (SIT) with GAD65 might alter the course of T1DM disease associated autoimmunity.

Following the molecular cloning of the human (3) and rodent GAD proteins (4) it was possible to carry out experiments with recombinant GAD65 to demonstrate that spontaneous diabetes in the NOD mouse (5, 6) was preventable. Of equal importance was the observation that diabetes was not inducible by GAD65 in rats (7) and mice (8). T1D therefore did not immediately seem to mimic other autoimmune diseases, which have animal models that are based on the actual immunization of an autoantigen. About 10 years after the demonstration that NOD mouse diabetes was preventable, Phase I (toxicity) and Phase II (safety) clinical trials of GAD65 have been completed. In the absence of adverse events among the GAD65 autoantibody positive Type 2 diabetes patients (so-called LADA patients or Latent Autoimmune Diabetes in the Adult) it may now be possible to move into Phase III clinical trials to test whether GAD65 SIT is efficacious.

The future approach to determine whether GAD65 SIT



is truly inducing immune tolerance, deviation, or both, is to carefully move from adult patients who already have diabetes such as LADA patients to new onset adult T1DM patients and then possibly to triple autoantibody positive first degree relatives since our ability to predict disease in such subjects is now well documented thanks to the DPT-1 study with parenteral insulin administration (9). The attempts to approach prevention in the fashion modeled in animals will by necessity require a carefully staked out pathway. Novel observations are particularly tantalizing as to the possibility to use GAD65 SIT and cause no harm in healthy subjects.

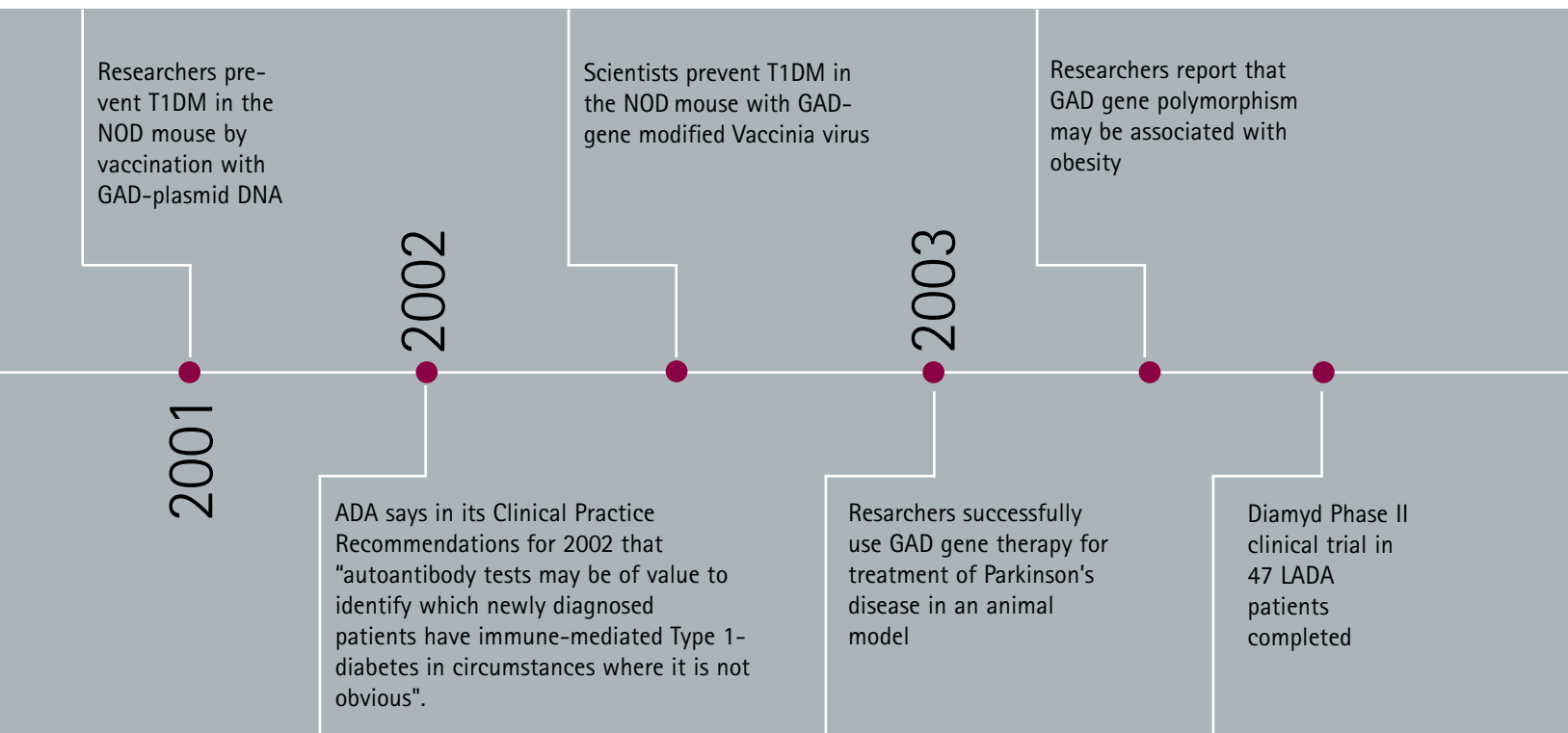
The beta cell expression of GAD65 in human beta cells is well established. There is a lack of qualitative and quantitative studies on GAD65 in the non-insulin producing endocrine islet cells. Also, we still do not fully understand the role of GABA for normal beta cell function. Recent observations suggest that increased body mass index (BMI) is associated with GAD65 autoantibodies in subjects not developing diabetes (10, 11). It will be critical to find out if subclinical GAD65 autoimmunity is related to obesity phenotypes arising because of altered beta cell function, insulin resistance, or both. Future studies will also require detailed studies of GAD65 gene expression in CNS areas controlling food intake since GAD65 gene polymorphisms affecting GABA production may be related to morbid obesity (*P. Boutin* and *P. Frougel*, personal communication). The role of GAD65 gene polymorphism in food intake regulation, obesity development and beta cell function may therefore need to be further studied before we can embark on large scale GAD65 SIT.

The relationship between Stiff Man Syndrome and GAD autoimmunity was critical to the demonstration that T1DM

sera immunoprecipitated 64K protein with GAD enzyme activity (12). We have now learned that the immune response to GAD65 in SMS is qualitatively and quantitatively different from T1DM and the approach to prevent either SMS, T1DM, or both may require different GAD65 SIT. One approach may be to base the SIT on the HLA association with the disease. In the Insulin Autoimmune Syndrome the autoantibody formation is strongly associated with HLA DRB1*0401. This haplotype is insufficient for the association with insulin autoantibodies in T1DM, which is rather associated with HLA DQA1*0501-B1*0201. Hence insulin as well as GAD65 is the autoantigen of more than one disorder of autoimmune character. Future studies will need to take these similarities and differences into account to design intervention trials that may lead to the identification of novel approaches to prevent or reverse autoantigen-specific autoimmunity. Don't sit around, GAD back to the future...

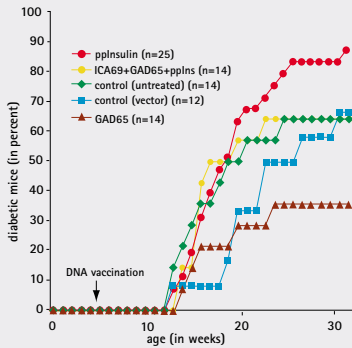
References

1. Peakman M, *et al.* Characterization of preparations of GAD65, proinsulin, and the islet tyrosine phosphatase IA-2 for use in detection of autoreactive T cells in Type 1 diabetes: report of phase II of the Second International Immunology of Diabetes Society Workshop for Standardization of T cell assays in Type 1 diabetes. *Diabetes* 50:1749-1754, 2001.
2. Mire-Sluis AR, *et al.* The World Health Organization International Collaborative Study for Islet Cell Antibodies. *Diabetologia* 43:1282-1292, 2000.
3. Karlens AE, *et al.* Cloning and primary structure of a human islet isoform of glutamic acid decarboxylase from chromosome 10. *Proc Natl Acad Sci US* 88:8337-8341, 1991.
4. Kaufman DL, *et al.* Autoimmunity to two forms of glutamate decarboxylase in Insulin-dependent diabetes mellitus. *J Clin Invest* 89:283-292, 1992.
5. Kaufman DL, *et al.* Spontaneous loss of T cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. *Nature* 366:69-72, 1993.
6. Tisch R, *et al.* Immune response to glutamic acid decarboxylase correlates with insulinitis in non-obese diabetic mice. *Nature* 366:72-75, 1993.
7. Bieg S, *et al.* GAD65 and insulin B chain peptide (923) are not primary autoantigens in the Type 1 diabetes syndrome of the BB rat. *Autoimmunity* 31:15-24, 1999.
8. Plesner A, *et al.* Immunization of diabetes-prone or non-diabetes-prone mice with GAD65 does not induce diabetes or islet cell pathology. *J Autoimmun* 11:335-341, 1998.
9. DPT-1: Effects of insulin in relatives of patients with Type 1 diabetes mellitus. *N Engl J Med* 346:1685-1691, 2002.
10. Rolandsen O, *et al.* Levels of glutamate decarboxylase (GAD65) and tyrosine phosphatase-like protein (IA-2) autoantibodies in the general population are related to glucose intolerance and body mass index. *Diabetologia* 42:555-559, 1999.
11. Weets I, *et al.* Male-to-female excess in diabetes diagnosed in early adulthood is not specific for the immune-mediated form nor is it HLA-DQ restricted: possible relation to increased body mass index. *Diabetologia* 44:40-47, 2001.
12. Baekkeskov S, *et al.* Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. *Nature* 347:151-156, 1990.



GAD in Graphs

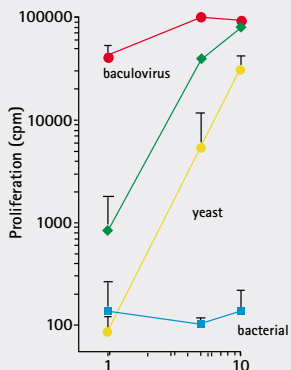
GAD65 DNA vaccination prevents diabetes in NOD mice



Karges *et al*, *Diabetes* 51:3237; 2002

- 4-6 week old female NOD
- Different DNA vaccinations
- Insulin construct does not protect from diabetes
- GAD65 construct prevents diabetes
- Combining constructs abolishes GAD65-induced protection

Baculovirus-encoded recombinant GAD outperforms bacterial and yeast GAD preparations in T cell proliferation assays



Peakman *et al* (2002) Characterization of preparations of GAD65, proinsulin and IA-2 for use in detection of autoreactive T-cells in Type 1 diabetes. *Diabetes* 50:1749-1754

- T cell recall immune responses were studied by proliferation assays using defined T cell lines and clones and different preparations of GAD65
- Two independently produced GAD65 preparations expressed by baculovirus gave higher proliferation indices than either yeast- or bacterial-produced GAD65

Untangling the GADs

Allan Tobin

My interest in GABA and GAD came from my interest in Huntington's disease – a degenerative neurological disorder that had killed the mother of two of my friends. I decided in the early 1980s that GAD was a reasonable “candidate gene” for Huntington's disease, and I set out to isolate it. The time was ripe for gene isolation since new molecular techniques were emerging every month – both new ways of making collections of genes and new methods for screening them for genes of interest.

Just at that time, *Dan Kaufman*, who had worked in my laboratory as a UCLA undergraduate and then moved to Berkeley to do graduate work, decided to move back to Los Angeles, to rejoin my laboratory, and to work on the brain.

Never suspecting that I would ever end up contributing to that field, I counseled Dan to work on a more tractable problem – the isolation of the GAD gene. At the time, few neuroscientists were using molecular techniques, and finding that gene would undoubtedly make a significant contribution to the field. Even if Huntington's disease is not caused by a mutation in GAD, I reasoned, having the gene in hand would accelerate progress in understanding the inhibitory circuits that are important in normal brain function and that fail in Huntington's disease.

Dan quickly succeeded in finding the GAD gene (which we now call GAD67), using a combination of immunological, molecular, and biochemical techniques. Pure luck – the ability of bacteria to make a functional GAD – allowed us to short circuit most of the slogging that we thought would be

necessary to prove we had the right gene. But there were some anomalies that later caused another graduate student, *Mark Erlander*, to challenge our initial view that “there is only one GAD”. Using a new set of techniques, in the early days of PCR (the polymerase chain reaction), Mark found another GAD gene, which we now call GAD65.

In 1990, Dan Kaufman, then a postdoctoral fellow at the Salk Institute, again returned to the lab, again interested in autoimmune disease. On the basis of a paper by *Pietro Di Camilli* on Stiff Man Syndrome – a rare complication of diabetes and of other conditions, Dan suspected that GAD65 or GAD67 might be identical to the unnamed 64 kD antigen first identified by *Åke Lernmark* in 1982 as the first target of Type 1 (T1D) antibodies. About the same time, *Steinunn Baekkeskov*, *Pietro Di Camilli*, and their colleagues came to a similar conclusion though they didn't know about GAD65, which we had only recently discovered. In collaboration with *Noel Maclaren* and *Mark Atkinson* at the University of Florida, we examined GAD autoimmunity in T1D patients, using GAD65 and GAD67 that we could produce from recombinant DNA. Autoantibodies to GAD indeed provided an

“our real excitement came from a thoroughly unexpected (and still unexplained) finding, that part of GAD65 showed a striking similarity to a piece of Coxsackievirus, a virus that is epidemiologically associated with Type 1 diabetes”

effective diagnostic or predictive test for T1D.

While autoantibodies are hallmarks of the autoimmune process and are valuable prediagnostic markers, autoreactive T cells, rather than autoantibodies, are likely to be the underlying cause of T1D. In further collaboration with Maclaren and Atkinson, we showed that newly diagnosed T1D patients, in contrast to healthy controls, have significant T cell responses to purified recombinant human GAD65.

We knew that these discoveries would be useful and important, but our real excitement came from a thoroughly unexpected finding – that part of GAD65 showed a striking similarity to a piece of Coxsackie virus a virus that is epidemiologically associated with T1D. This sequence match suggested a possible mechanism for the initiation of an immune response. We thought that T1D might involve “molecular mimicry,” in which the Coxsackie viral antigen triggered an immune response that cross-reacted with GAD. With this highly speculative hypothesis of molecular mimicry in mind, we set about to look for evidence that GAD autoimmunity might actually be pathogenic rather than merely diagnostic in T1D.

To test the hypothesis that an autoimmune

response to GAD65 initiates autoimmunity, Dan Kaufman and our other UCLA colleagues turned to the non-obese diabetic mouse (NOD mice).

Early treatment with GAD65 induced tolerance not only to GAD65 itself, as expected, but also prevented the development of autoimmunity to other β cell antigens as well.

Still more amazingly, mice made tolerant to GAD65 never developed insulinitis or diabetes. This was our first strong indication that GAD-specific autoimmunity may be important in the pathogenesis of T1D. This finding, together with subsequent work by Dan Kaufman and others, was the basis for the clinical trial now being conducted by DIAMYD MEDICAL.



Allan Tobin, Ph.D., is a Professor and Director of the Brain Research Institute at UCLA, Los Angeles. Tobin is also Scientific Director of the Hereditary Disease

Foundation, which participated in the identification of the gene that causes Huntington's disease. Tobin has specialized in the use of molecular methods for synthesis, function and breaking down of GABA. GABA provides a significant inhibitory signal in the brain and the pancreas.

In Nature, Anything that Can Happen Does Happen

Dan Kaufman

I did not want to clone GAD. It was the mid-1980's, and I was first year graduate student of *Allan Tobin* at UCLA. New cloning technologies had just been developed that could allow neurobiologists to clone rare brain mRNAs. Allan and I thought that some of the most important genes in brain development and disease would be neurotransmitter receptors and neurotransmitter synthesizing proteins. I wanted to use antibodies to screen brain cDNA expression libraries for GABA receptors, or use serum from multiple sclerosis patients to identify the protein targets of their autoimmune response. Allan, however, knew that there was a good antibody available against GAD (developed by *Oertel*) and instructed me to go after GAD.

I constructed and screened a brain cDNA expression library and quickly isolated one immunoreactive clone. In those days, there was great contention about the molecular weight of GAD, so I tested the putative GAD clone for its GAD enzymatic activity. Luckily, the one immunoreactive clone I had isolated encoded an enzymatically active protein when expressed in *E. coli*. Our report describing the cloning of GAD (*Science*, 1986) showed that expression libraries could be screened for functional activity, led to a greater understanding GAD biochemistry, and provided neuroscientists with a cDNA probe for studying GAD and GABA in brain development and disease.

My discussions with a new graduate student in the Tobin lab, *Mark Erlander*, led us to suspect that another GAD gene existed, despite our failure to detect another gene by low stringency screening. Using degenerate PCR techniques, Mark was the first to isolate the second GAD gene, which encoded a 65 kD protein (GAD65). The clone that I had isolated was renamed GAD67, based on its molecular weight.

Using recombinantly produced GAD67 protein, I next generated a GAD67-specific antibody. This antibody has been widely used by neuroscientists to study GABAergic neurons. Using this antibody together with a GAD65-specific antibody, I showed for the first time that while GAD67 is distri-

buted throughout the neuron, GAD65 is localized in the nerve axon terminals. While most of GAD67 is saturated with its cofactor, less than half of GAD65 exists as active holoenzyme. We suggested that the modulation of GAD65 apo/holoenzyme levels in nerve terminals could couple GABA production to neuronal activity (*J. Neurochem*, 1991).

My change in course to study Type I diabetes (T1D) resulted from my car breaking down. I was then a postdoc at the Salk Institute in San Diego. After working in the lab and finding that my car wouldn't start, I decided to pass time in the library. I picked up the latest issue of *Lancet*, and it fell open to a report entitled "64,000 Mr autoantibodies as predictors of insulin-dependent diabetes" by *Mark Atkinson* and colleagues. A then little known factlet came to mind that besides the brain, GAD was also expressed in the cells that made insulin. From my work in the Tobin lab, I knew that there were two forms of GAD, both of which could be candidates for this 64K autoantigen.

I learned that the 64K autoantigen was a long sought after β -cell antigen described by *Steinunn Baekkeskov* and *Åke Lernmark*. In addition, *Michele Solimena* and *Pietro De Camilli* had just reported an association between GAD autoimmunity and Stiffman syndrome. However, they also reported that they did not detect GAD autoimmunity in T1D patients, which in retrospect, was missed only because the GAD antibodies in T1D patients could not recognize the denatured GAD epitopes in their assay system. Despite this reported lack of association, I decided that it was worth a try to test whether T1D sera could recognize recombinant GAD67 or GAD65. This would be an easy experiment using the recombinant GADs available in the Tobin lab. I was again fortunate, since at this time, the existence of two GADs was only known, and their cDNAs only available, in the Tobin lab.

I got permission from Allan to do an immunoprecipitation experiment in his lab over the next weekend. I got T1D sera from *Michael Clare-Salzler*, and later, from *Mark Atkinson* and *Noel Maclaren*. Using the recombinant GADs, I quickly



Daniel Kaufman, Ph.D., is a Professor, active within the department of Molecular and Medical Pharmacology at the UCLA School of Medicine in Los Angeles. In a paper in November

1993, Kaufman demonstrated that the administration of GAD to mice that would otherwise develop insulin-dependent diabetes prevented the outbreak of this disorder.

Kaufman was also a member of the group associated with Allan Tobin, which was the first to submit a patent application for the full cDNA code for GAD, the application that Diamyd Medical licenses. Kaufman's sphere of interest is focused on GAD and its relation to diabetes.

found that sera from T1D patients contained GAD autoantibodies that were primarily directed against GAD65. Using recombinantly produced fragments of GAD65, I was able to map some of the autoantibody epitope recognition patterns. These were the first T1D diagnostics based on using recombinant GADs. I had also read about an epidemiological association between Coxsackie virus and T1D.

When we compared Mark Erlander's GAD65 DNA sequence with other sequences in the DNA sequence data banks, we found a large sequence similarity with a Coxsackie virus. This was a very provocative finding, which could explain how β -cell autoimmunity was started, or augmented.

We submitted a manuscript with our findings to *Science* essentially at the same time as Steinunn Baekkeskov and colleagues submitted their findings to *Nature*. Unfortunately, a reviewer insisted that we provide further proof of GAD-Coxsackie virus molecular mimicry before it could be published. Consequently, we were not able to publish our work until after that of Baekkeskov and colleagues. While very disappointing at the time, this may have been in a way fortuitous – I had just become a new Assistant Professor at UCLA, and it prompted me to go after the next major question – what was the role of GAD in T1D?

As T1D is thought to be T cell, and not autoantibody mediated, it was crucial to understand T cell responses to β -cell antigens. I was again extremely fortunate to have *Jide Tian* join my new lab, who was highly knowledgeable and skilled in cellular immunology. Using the NOD mouse model of T1D, Jide found that a Th1-type response first arose to GAD65, and then spread intra- and inter-molecularly to other β -cell antigens. Jide treated very young NOD mice with GAD65 or other β -cell antigens in a way that inactivated reactive T cells. The mice that were tolerized to GAD65 had no autoimmune responses or insulinitis, while those tolerized to other antigens did display β -cell autoreactivity (*Nature*, 1993). Thus, the early inactivation of GAD65 reactive T cells could circumvent the development of autoimmunity, qualifying GAD65 as a key antigen in the induction of murine T1D.

We next examined what could be done to inhibit an autoimmune process after it had already started. Jide showed that injecting GAD65 in an adjuvant that induced Th2 responses to GAD65 could greatly inhibit the progression of the autoimmune process in NOD mice that already had

“These and subsequent experiments suggested that the greater protective effect of GAD65 was due to its greater ability to induce Th2 responses. It is these experiments that led to the GAD65 vaccine that *Diamyd* is testing in clinical trials”

an established autoimmune process. Moreover, GAD65 vaccination could also protect transplanted islets in diabetic mice, significantly better than the other β -cell autoantigens (*Nature-Medicine*, 1996). These and subsequent experiments suggested that the greater protective effect of GAD65 was due to its greater ability to induce Th2 responses. It is these experiments that led to the GAD65 vaccine that *DIAMYD* is testing in clinical trials.

We also began to examine the possibility of T cell cross-reactivity between GAD and Coxsackie virus. Jide showed that T cell cross-reactivity did occur, both at the peptide level and at the whole protein level between GAD65 and the Coxsackie virus protein – but only in mice with the diabetes susceptible MHC II allele, and not in mice with other MHC II alleles (*J. Exp. Med.*, 1994). The jury is still out as to whether the epidemiological association of Coxsackie virus with T1D is due to the ability of Coxsackie virus to infect islet cells, or due to T cell cross-reactivity with GAD. As it is generally found that in nature, anything that can happen does happen, it would not be surprising to find that both mechanisms can occur, and in some cases, initiate or augment β -cell autoimmunity.

It has been most gratifying to see our work at the bench contribute to new diagnostics and potential treatments for diabetes, as well as neurological diseases. Our work has been highly dependent on the support of Allan Tobin, as well as contributions by Jide Tian, Michael Clare-Salzler, Mark Atkinson and *Paul Lehmann* – who have also had the patience of saints in teaching me immunology.

References

1. Baekkeskov, S, *et al*, Antibodies to a Mr 64,000 human islet cell antigen precede the clinical onset of insulin-dependent diabetes. *J. Clin. Invest.* 79, 926-934 (1987).
2. Baekkeskov, S, *et al*, Revelation of specificity of 64k autoantibodies in IDDM serums by high-resolution 2D-gel electrophoresis. Unambiguous identification of 64k target antigen. *Diabetes* 38, 1133-1141 (1989).
3. Christgau, S, *et al*, Pancreatic β -cells express two autoantigenic forms of glutamic acid decarboxylase, a 65kDa hydrophilic form and a 64kDa amphiphilic form which can be both membrane-bound and soluble. *J. Biol. Chem.* 266, 21257-21264 (1991).
4. Baekkeskov, S, *et al*, Identification of the 64k autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. *Nature* 347, 151-156 (1990).
5. Kim, J, *et al*, Higher autoantibody levels and recognition of a linear N-terminal epitope in the autoantigen GAD65 distinguish stiff-man syndrome from insulin dependent diabetes. *J. Exp. Med.* 180, 595-606 (1994).
6. Kanaani, J, *et al*, A combination of three distinct trafficking signals mediates axonal targeting and presynaptic clustering of GAD65. *J. Cell Biol.* 158, 1229-1238. (2002).
7. Kash, S. F., *et al*, Increased anxiety and altered responses to anxiolytics in mice deficient in the 65 kDa isoform of glutamic acid decarboxylase. *Proc. Natl. Acad. Sci.* 96, 1698-1703 (1999).
8. Shi, Y., *et al*, Increased expression of glutamic acid decarboxylase and GABA in pancreatic β cells impairs glucose-stimulated insulin secretion at a step proximal to membrane depolarization. *Am. J. Physiol. Endocrinol. Metab.* 279, 684-694, (2000).
9. Schwartz, H., *et al*, High resolution epitope mapping and structural modeling of human glutamic acid decarboxylase 65. *J. Mol. Biol.* 287, 983-999 (1999).
10. Jaume, J. C., *et al*, Suppressive effect of GAD65-specific autoimmune B lymphocytes on processing of T cell determinants located within the antibody epitope. *J. Immunology* 169, 665-672, (2002).

Contributions to the GAD65 Field

Steinunn Baekkeskov

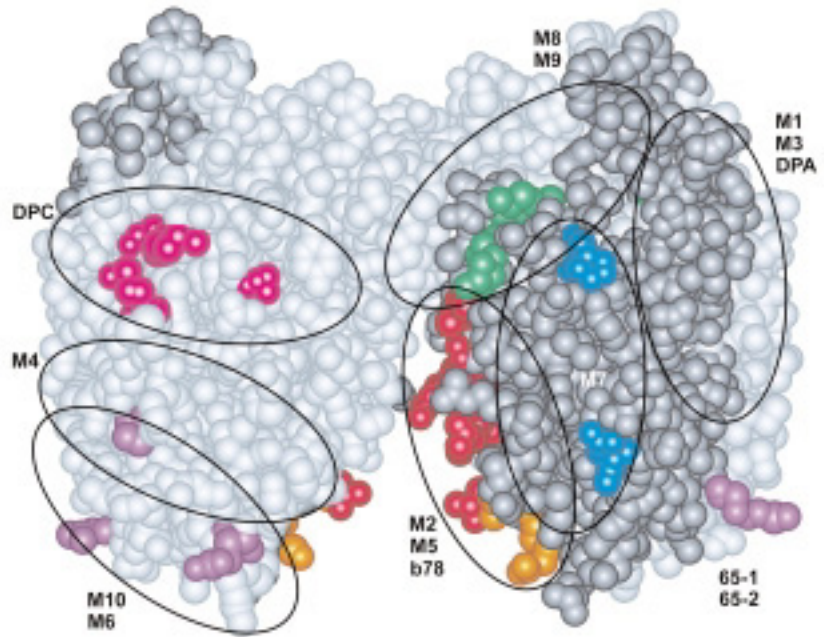
In the 1960's and 1970's several investigators discovered evidence to show that insulin dependent diabetes mellitus (Type 1 diabetes or T1D) is an autoimmune disease characterized by circulating autoantibodies to the β cell in human pancreatic islets and infiltration of lymphocytes into the islets. However, the target antigen of this autoimmunity was completely unknown, when I joined the Hagedorn Laboratory in Copenhagen in late 1979 to work with Åke Lernmark. Using expertise I had gained in studying target antigens in trypanosomes, I developed a method that allowed a very sensitive detection of human β cell proteins that bound to circulating autoantibodies in the blood of patients. To our surprise, we discovered that autoantibodies in the blood from 8 out of 10 diabetic children bound to the same protein, a 64kD antigen from human islets while none of 10 healthy children recognized this protein. This was the very first indication that there was a specific target antigen in the autoimmune response in T1D (1). We then showed that the autoantibodies to the 64kD protein can be present many years before clinical onset of T1D (2), suggesting that these autoantibodies can be used as a sensitive marker to identify individuals at risk of developing T1D. Also, in all autoimmune diseases the target antigen is of critical importance because of its potential to be used to prevent the autoimmune

disease. Having established my own laboratory first at the Hagedorn Research Laboratory and later at the University of California, San Francisco, we developed a method for partially purifying the 64kD protein (2-3). We characterized important biochemical and biophysical parameters of the antigen (2-4) in a painstaking effort that culminated in its identification as the smaller isoform of the GABA-synthesizing enzyme glutamic acid decarboxylase or GAD65 (5). The identification of a component of the 64kD autoantigen as GAD65 transformed the field of Immunology of Diabetes, because it became possible to use recombinant protein for development of autoantibody assays and for studies of autoimmune mechanisms in the NOD mouse. A few years later, a second component of the 64kD autoantigen, left behind by our purification method, was identified as the protein IA-2 by Michael



Steinunn Baekkeskov, Ph.D., is a Professor at the University of California. Baekkeskov's current research projects focus on four main areas: i) structure, function, and cell biology of GAD65, IA-2 and glim 38; ii) characterization of disease specific B-cell epitopes in GAD65, and IA2, and the temporal pattern of their recognition during early and late phases of β -cell destruction; iii) characterization of autoimmune T cell epitopes in GAD65 and IA2, and how they can be used to induce apoptosis in autoimmune T cells during the preclinical phase to prevent disease; iv) and mechanisms of transplantation tolerance, and development of methods to prevent allo- as well as autoimmune destruction of islet cell transplants.

GAD65 molecule



Christie, my first postdoctoral fellow, in his own lab. Together, GAD65 and IA-2, which are expressed in both β cells and neurons, are recognized by 80-90% of T1D patients. In neurons, GAD65 is also a target of autoimmunity in a rare neurological disorder Stiff-man syndrome (6).

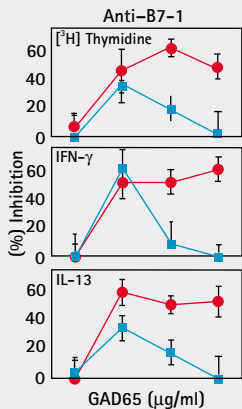
Following the discovery of GAD65 as a major target autoantigen in T1D (5), my lab has focused on understanding the structure, cell biology, and function of this molecule, and how it is recognized by the immune system. The subcellular trafficking of GAD65 is the parameter that most clearly distinguishes it from the highly homologous GAD isoform, GAD67, and is dependent on unique trafficking signals in the N-terminal region (7 and refs therein). By knocking-out GAD65 in the mouse, we showed that GABA generated by GAD65 is involved in fine tuning of inhibitory neurotransmission in response to a variety of environmental stimuli (8 and refs therein), while overexpression of the protein in β cells revealed a role of GABA in negative regulation of first phase insulin secretion (9). Fine mapping of the autoimmune epitopes recognized by GAD65 autoantibodies in human patients revealed that they target almost the entire surface of the molecule (10 and refs therein) and led to the first

The figure is printed with permission from Academic Press (*Journal of Molecular Biology*, Vol.287, No 5, April 16, 1999, pp. 983-999, Baekkeskov S, Schwartz HL).

3D model of the GAD65 dimer (10). This structural information enabled us to show how the epitope specificity of autoimmune B cells, a critical player in GAD65 presentation to T cells and development of T1D, influences the autoimmune T cell epitope repertoire in the protein (11).

GAD in Graphs

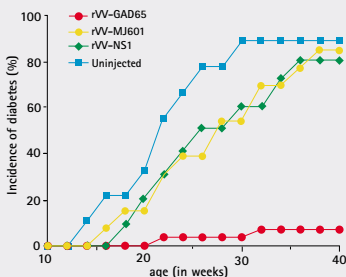
In Type 1 diabetes patients, GAD65-reactive T cells are primarily memory T cells



Viglietta *et al* (2002) GAD65-reactive T cells are activated in patients with autoimmune Type 1a diabetes. *J Clin Invest* 109:895-903.

- T cell recall immune responses were studied by proliferation and cytokine assays using GAD65 and inhibition of the costimulatory molecule B7.1
- T cells from Type 1 diabetics were not inhibited by anti-B7.1, while those from controls were. This indicates that the Type 1 diabetic T cells are of a memory T cell phenotype.

Treatment of young NOD mice with rrVV-GAD65 prevents diabetes development



Sun *et al*. (2002). Prevention of autoimmune diabetes by immunogene therapy using recombinant vaccinia virus expressing glutamic acid decarboxylase. *Diabetologia* 45:668-676

- 3 week old female NOD mice were injected with vaccinia constructs coding for GAD or control antigens
- Mice receiving the GAD65 construct were protected from diabetes

The GAD65 Stanford Perspective

Hugh McDevitt

Initial work with GAD65 identified it as one of the first to elicit a spontaneous immune response in 3-4 week old NOD mice. Next, we used GAD65 to suppress or delay the diabetic process. Treatment with 4 doses of GAD65 intravenously at 12 weeks of age resulted in a large decrease in diabetes incidence at 30 weeks. Other islet cell proteins, or foreign proteins had no effect.

Transgenic mice with T Cell Receptors (TCRs) for the 206 and 286 peptides, and a hybrid TCR transgenic with the 221 α chain and the 286 β chain, revealed that in all 3 TCR transgenic NOD lines neither insulinitis nor Type 1 diabetes developed. T cells in all 3 lines are reactive to cognate peptide and produce IL-2, IFN γ , IL-10, and some TNF α . The T cells are positively selected in the thymus, but only escape negative selection via the expression of a second α chain. The 206 and 286 TCR transgenic lines on the C α ^{-/-} background had near complete deletion of T cells. MHC tetramer analysis of 286 TCR affinity of the 286 TCR showed that it was of extremely low affinity.

T cells were partially negatively selected in the thymus, and underwent further negative selection in the periphery. Only 10-20% of peripheral CD4⁺ T cells are 286 tetramer positive, while 30-40% of CD8⁺ T cells are tetramer positive. Cell transfer experiments showed that these mice have T cells capable of partially suppressing, and definitely delaying the onset of Type 1 diabetes in a standard transfer system.

To date, none of these mice have developed diabetes or insulinitis. Crosses with a RIP-HuGAD65 high expressing transgenic NOD line has not resulted in diabetes or insulinitis in either the 206 or 286 lines. Other studies are currently underway. However, it appears that in all 3 TCR transgenic

mouse lines, GAD65 specific T cells are protective.

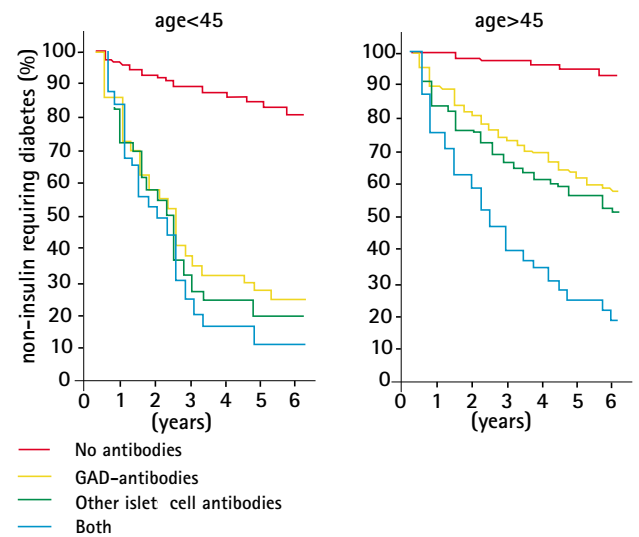
A review of the literature shows that most GAD65-specific T cell clones either do not cause diabetes or insulinitis, or in some cases are protective. (One clone, reported by *Robert Sherwin*, did cause Type 1 diabetes.) Further, *von Boehmer (J. Exp. Med., in press)* has shown that induction of recessive tolerance to GAD65 with a transgene insuring high level GAD65 expression in endosomal compartments does not change Type 1 diabetes incidence.

These results suggest that GAD65 is primarily a protective antigen in Type 1 diabetes in NOD mice. Whether it is also a protective antigen in patients is an open question. Nonetheless, experiments from our and other laboratories suggest that administration of GAD65 in non-inflammatory vehicles result in a delay in onset, or a decrease in incidence of NOD diabetes. This raises the question of whether GAD65 is a protective islet cell antigen because of its unusual location in β cells (in vesicles similar to neuronal vesicles), with this presentation resulting in a preferential elicitation of a TH2 response to this islet cell protein. This increase in TH2 T cells in the islets may then be a factor in delaying onset of diabetes and/or decreasing its incidence.



Hugh McDevitt, MD, is Professor of the Department of Microbiology and Immunology at Stanford University School of Medicine, with a joint appointment in the Department of Medicine.

Following his discovery of linkage of the major histocompatibility complex with specific immune responses, McDevitt has pursued the mechanisms of this association over the past several decades, and most recently has been studying the immune response of NOD mice to GAD65.



The Search for Islet Antigens

David Leslie and Marco Londei

The search for islet antigens associated with the immune response which precedes and predicts the clinical onset of Type 1 diabetes received a substantial boost with the identification of GAD as one of the major antigens involved in the aberrant immune response. We used a large cohort of identical twins which had been followed prospectively for several years to show for the first time that in these twins GAD antibodies were highly predictive of progression to clinical diabetes. Further studies found that the isotypes of these GAD antibodies were largely restricted to a single isotype IgG1. In contrast, patients with a rare nerve disease Stiff Man Syndrome, who also have GAD antibodies, and in whom about half the cases also have diabetes, were found to have a broad isotype profile involving antibody isotypes other than IgG1. These observations suggested that the antibody immune response to GAD in Type 1 diabetes showed maturation at an early stage and was distinct from that found in Stiff Man Syndrome. We showed that the T cell responses to GAD also differed in Type 1 diabetes as compared with normal subjects and patients with Stiff man syndrome. Specifically, the dominant T cell response in diabetes was to peptide epitopes (fragments) at the carboxy-

“ *These studies, taken together, suggest a distinct mature immune response to GAD involving cellular and humoral immunity in Type 1 diabetes* ”

terminal end of the GAD molecule. These studies, taken together, suggest a distinct mature immune response to GAD involving cellular and humoral immunity in Type 1 diabetes and raised the possibility that modulation of that response might modify the destructive immune process associated with progression to insulin dependence.

The potential to modify that GAD immune response and thereby modify the disease course was illustrated by others in animal models and is now being tested in patients with autoimmune diabetes. Should these studies prove successful the next stage would be to consider intervention in at-risk subjects since GAD antibodies, allied to other disease markers, are highly predictive of disease progression.



David Leslie, MD, FRCP, is Professor of Diabetes and Autoimmunity at the Royal London and St. Bartholomew's School of Medicine, University of London.

Leslie has been involved in diabetes research and clinical studies since 1975. Leslie has been Director of the British Diabetic Twin Study since 1982, the world's largest twin study of its type. By studying twins Professor Leslie has been able to show the possibilities for predicting and preventing diabetes.



Marco Londei, MD, is a Professor at the Imperial College School of Medicine in London, is one of Great Britain's most internationally renowned scientists in the field of autoimmunity. Londei's research has been concentrated on T cells in autoimmunity.

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GAD Peptide Vaccines for T1DM: Not Just a Blueprint?

J.-M. Bach

Type 1 diabetes is an autoimmune disease resulting in the destruction of pancreatic islet β -cells, which secrete insulin, by autoreactive T lymphocytes.

One of the major challenges of the new century will be the treatment of organ-specific autoimmune disease. For Type 1 diabetes, it means disease prevention before the complete destruction of the β -cell mass, or replacement of insulin-secreting cells after their disappearance. Pancreas and islet cell transplantation are effective β -cell replacement therapy for diabetes, but the shortage of human donor pancreata has led to search for potential alternative sources of islet cells (pig islets, *in vitro* generation of β -cells from pancreatic duct cells or from stem cells). Beside replacement approaches, diabetes prevention implicates the development of immunotherapies, especially specific immunomodulations of auto-antigens involved in Type 1 diabetes pathogenesis.

Immunotherapies are also necessary for extensive β -cell replacement approaches, to block the autoimmune process and to avoid strong immunosuppressor treatments. To be attractive, immunotherapies of Type 1 diabetes have to produce a long-lasting protective response specific for self-antigens.

On one hand, specific modulations and tolerance could be obtained in rodent models with whole self-proteins or DNA encoding auto-antigens. On a second hand, synthetic peptide-based vaccines could be a very attractive approach for specific immunomodulations, permitting to target clonal elimination or unresponsiveness of aggressive T cells. Peptides can be produced in large quantities with high purity at low cost, and are safer than recombinant proteins. To allow the design of antigen-specific immune therapies targeting pathogenic autoreactive T cells, we need to characterize peptide specificities of autoreactive T cells, which should also provide fabulous markers of islet cell damage, useful for therapeutic trials and diagnosis of individuals with increased risk for disease, and could help to unravel the pathophysiology of autoimmune diabetes.

Several self-antigens have been identified in Type 1 diabetes. Among them, GAD (Glutamic Acid Decarboxylase) is a crucial early target involved in diabetes in human and non-obese diabetic (NOD) mouse. Its role is considered presently as decisive in autoimmunity initiation as well as in progression to overt disease. A majority of studies of diabetes autoimmunity process and prevention are focused on this autoantigen. In rodent models, the role of a peculiar set of T lymphocytes, expressing the CD8 marker, in β -cell aggression is not clearly understood. CD8⁺ T cells could be implicated not only in diabetes induction, but also in progression to destructive insulinitis and overt diabetes. Teams of *E. Sercarz* (1) and *A. Cooke* (2) have identified two peptides derived from GAD and recognized by CD8⁺ T lymphocytes in NOD mouse (peptide 546 and 515, respectively). CD8⁺ T cells specific for these peptides are detected early in young NOD mouse. Our squad have very recently specific another GAD-derived peptide (peptide 90), which may be crucial in diabetes progression in mice [3]. For the first time, we showed that CD8⁺ T cells specific for a GAD-derived peptide are implicated in diabetes aggravation. Specific cytotoxic CD8⁺ T cells were activated and could play an important role in progression of insulinitis to overt disease. We presently test immunoprevention of spontaneous diabetes in NOD mice using CD8⁺ T cell-inducing peptides.

And in humans? In humans, while autoantibody responses have been extensively characterized, very little is known of the natural history of T cell autoreactivities, neither for CD8⁺ T cells (probably the first T lymphocytes to colonize pancreatic islets), nor for T lymphocytes expressing CD4 antigen. We and others have already identified GAD-derived peptides presented to CD4⁺ T lymphocytes of recent-onset diabetic patients [4-6]. In humans, we need also to identify relevant CD8⁺ T cell-inducing peptides of GAD. In the future, discovery of GAD-peptides recognized by self-reactive aggressive CD8⁺ T lymphocytes and elucidation of key immunological events leading to diabetes initiation or aggravation in humans may permit to define efficient peptide-vaccine strategies.

References

1. Quinn A, *et al*. MHC class I-restricted determinants on the glutamic acid decarboxylase 65 molecule induce spontaneous CTL activity. *J Immunol*. 2001;167:1748-57.
2. Bowie L, *et al*. Generation and maintenance of autoantigen-specific CD8⁺ T cell clones isolated from NOD mice. *J Immunol Methods*. 1999;228:87-95.
3. Gauvrit A, *et al*. Identification of a relevant GAD65 H-2Kd-restricted epitope recognized by CD8⁺ T cells in NOD mice. 2003. *Unpublished data*.
4. Bach JM, *et al*. High affinity presentation of an autoantigenic peptide in Type 1 diabetes by an HLA class II protein encoded in a haplotype protecting from disease. *J Autoimmun*. 1997.
5. Bach JM, *et al*. Identification of mimicry peptides based on structural motifs of epitopes derived from 65-kDa glutamic acid decarboxylase. *Eur J Immunol*. 1998;28:1902-1910.
6. Endl J, *et al*. Identification of naturally processed T cell epitopes from glutamic acid decarboxylase presented in the context of HLA-Dr alleles by T lymphocytes of recent onset IDDM patients. *J Clin Invest*. 1997;99:2405-2415.

J.-M. Bach, Ph.D., D.V.M., is Assistant Professor in Endocrinology and Director of the Immunoendocrinology Unit of Nantes, France. Doctor Bach has specialized in the study of the cellular immune response to GAD in Type 1 diabetes (mouse, dog and human).

No GAD No Type 1 Diabetes?

Ji-Won Yoon, Hee-Sook Jun and Julia McFarlane

The prevention of Type 1 diabetes has long been a primary area of research for our laboratory. Several β cell autoantigens have been implicated in the triggering of β cell-specific autoimmunity, and GAD65 is a strong candidate in both humans and the diabetes-prone nonobese diabetic (NOD) mouse, which is one of the best animal models for human autoimmune diabetes. In the NOD mouse, GAD, as compared with other β cell autoantigens examined, provokes the earliest T cell proliferative response.

We hypothesized that GAD expression in the β cells may be important for disease initiation. To address this, we took a transgenic approach and selectively suppressed GAD expression in the β cells of NOD mice. We found that complete suppression of GAD expression in the β cells of anti-sense GAD transgenic mice ([SJL \times C57BL/6] F2 mice) backcrossed with NOD mice resulted in the prevention of autoimmune Type 1 diabetes. These results support the hypothesis that GAD may play an important role in the development of T cell-mediated autoimmune diabetes. However, we cannot exclude the possibility that diabetes-resistance genes from the founder mice may have been transmitted to the anti-sense GAD transgenic mice. To address this issue, we are presently examining the development of insulinitis and diabetes in β cell-specific GAD knock-out NOD mice to confirm the role of GAD in the initiation of Type 1

autoimmune diabetes.

Several different approaches for immune therapy using GAD have been tried for the prevention of diabetes in animal models. We used a recombinant vaccinia virus (rVV) expressing GAD as a vaccine, as rVVs can induce humoral and cell-mediated immune responses to target proteins and the induced immune responses can be long lived. We were able to show that administration of GAD-expressing rVV effectively prevented autoimmune diabetes in an age- and dose-dependent manner through active suppression of effector T cells in NOD mice. Although the therapeutic effect of GAD-based immunotherapy is different depending on the route of administration, experimental conditions, and quality of antigen, treatment using GAD may be of importance with regard to future strategies for the prevention of Type 1 diabetes in humans.



Dr. Ji-Won Yoon holds a Canada Research Chair in Diabetes and is the director of the Laboratory of Viral and Immunopathogenesis of Diabetes in the Faculty of Medicine, University of Calgary. Previously, Yoon spent 10 years as a senior investigator at the National Institutes of Health. Over the past 5 years, Yoon has been involved in studies on the role of glutamic acid decarboxylase (GAD) in the pathogenesis of diabetes and the development of methods to prevent autoimmune diabetes by immunogene therapy using recombinant vaccinia virus vectors expressing GAD.

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What is the Genetic Basis of T1DM?

Michael Clare-Salzler

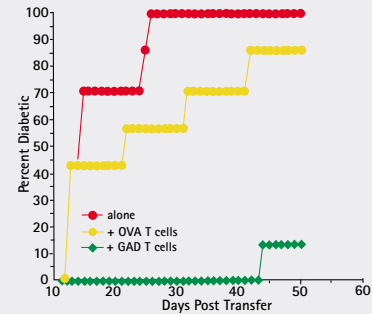
The goal of my research is to establish the cellular, molecular, and genetic basis for the immunopathogenesis of Type 1 diabetes (T1D) and to develop methods to prevent this disease. I have concentrated on identifying autoantigens and determining the role of antigen presenting cells, e.g. dendritic cells (DC), in T1D. Our publications were among the first defining glutamate decarboxylase (GAD) as an autoantigen. Our subsequent studies demonstrated GAD administration prevented disease in diabetes-prone NOD mice. Recently, the NIH-

based diabetes prevention study, TrialNet, developed plans for a trial to test whether GAD administration prevents T1D in humans.

Michael Clare-Salzler, MD, Professor of Pathology, Immunology and Laboratory Medicine, University of Florida, Director of Research and Academic affairs, research; part of the UCLA team that characterized GAD as an autoantigen in NOD mice, demonstrated GAD responses in NOD mice, study of the role antigen presenting cells in Type 1 diabetes, first to demonstrate a tolerogenic role for dendritic cells in the autoimmunity of the NOD mouse, first lab to demonstrate abnormal regulation Cox-2 regulation and prostaglandin metabolism in Type 1 diabetes.

GAD in Graphs

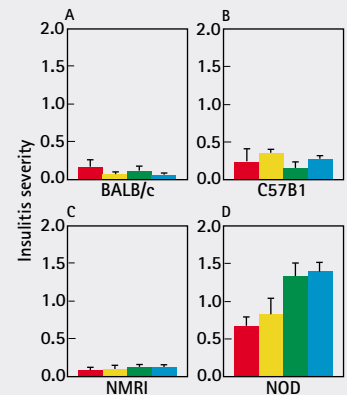
Induction of GAD65-specific regulatory T cells modulates diabetes in NOD mice



Tisch *et al.* (1998) Induction of GAD65-specific regulatory T cells inhibits ongoing autoimmune diabetes in nonobese diabetic mice *Diabetes* 47:894-899

- Irradiated NOD mice develop diabetes when diabetogenic splenic cells are transferred alone or cotransferred with OVA-specific T cells.
- Cotransfer of GAD-specific regulatory T cells prevents diabetes development

Immunization with GAD65 does not induce murine diabetes



Plesner *et al.* (1998) Immunization of diabetes-prone or non-diabetes-prone mice with GAD65 does not induce diabetes or islet cell pathology *J Autoimmunity* 11:335-341

- 11 wk old NOD mice were injected twice sc with 75mg GAD65 or BSA.
- Non-diabetes prone mice (Balb/c, C57/B1 and NMRI) did not develop diabetes.
- NOD mice injected with GAD65 developed less insulinitis

GAD65 and the Immunoregulation of Autoimmune Diabetes

Roland Tisch

Our group has used GAD65 as a model β cell autoantigen for the purpose of developing strategies of antigen-specific based immunotherapy, and defining events involved in the breakdown of self-tolerance within the T cell compartment in nonobese diabetic (NOD) mice. As in Man, GAD65-specific reactivity can be detected in NOD mice early in the diabetogenic response, suggesting a key role for this β cell autoantigen in both murine and human Type 1 diabetes (T1D).

The goal of antigen-specific based immunotherapy is to selectively prevent and/or suppress pathological autoimmunity without hindering the “normal” function of the immune system. In a number of models of autoimmunity including T1D, prevention and/or treatment of disease is typically accomplished through induction of so-called immunoregulatory T cells, of which there appear to be a number of “types” exhibiting various physical and functional properties. We and others have found that administration of GAD65 protein or peptides via different routes to young NOD mice can prevent initiation of the diabetogenic response. More importantly, administration of GAD65 can prevent the onset of overt diabetes even at late stages of preclinical T1D in NOD mice (1-4). This latter scenario is reflective of a clinical setting in which prediabetic individuals exhibiting ongoing β cell autoimmunity would be considered for some form of intervention. Furthermore, we have recently found that GAD65 vaccination can also be effectively applied to induce islet graft tolerance. Specifically, islets transplanted into diabetic NOD mice are protected long-term via administration of genetic vaccines encoding a portion of GAD65 and cytokines known to promote T cells with immunoregulatory function. One key characteristic of the

protection mediated by GAD65 administration is that infiltration of T cells and other immune cells into the islets is efficiently blocked regardless of the stage of disease progression. This property appears to be unique to GAD65 since protection mediated by administration of other β cell autoantigens is usually marked by continued infiltration of the islets, especially when the diabetogenic response is ongoing at the time of treatment. Currently, there is a need to further define the properties of GAD65-specific immunoregulatory T cells. For example, different “types” of T cells exhibiting distinct as well as overlapping immunoregulatory functions appear to be induced by administration of GAD65 under the appropriate conditions. Therefore, the general immunotherapeutic efficacy of GAD65 vaccination may reflect the relative number and “variety” of immunoregulatory T cells elicited by treatment.

Indeed, GAD65-specific T cells may “normally” regulate the progression of T1D. We have established NOD mice in which a significant frequency of developing T cells express a “transgenic” T cell receptor (TCR) specific for a peptide derived from GAD65. T cells expressing the transgenic TCR are efficiently deleted through mechanisms that prevent the development of autoreactive T cells and maintain tolerance to self-proteins such as GAD65. Interestingly, T1D is exacerbated in these transgenic NOD mice. This finding suggests that deletion of T cells expressing the transgenic TCR results in the loss of GAD65-specific T cells which regulate the diabetogenic response. In the context of immunotherapy, GAD65 vaccination may in part lead to the expansion of this pool of immunoregulatory T cells. The task at hand is to determine whether findings regarding the role of GAD65-specific immunoregulatory T cells in NOD mice can be extrapolated to diabetic individuals and/or those at high risk and exploited for the purpose of immunotherapy.

References

1. Tisch, R., *et al.*, Induction of GAD65-specific regulatory T cells inhibits ongoing autoimmune diabetes in nonobese diabetic mice. *Diabetes*, 47:894, 1998.
2. Tisch, R., *et al.*, Induction of GAD65-specific Th2 cells and prevention of autoimmune diabetes at late stages of disease development is epitope dependent. *J Immunol*. 163:1178, 1999.
3. Tisch, R., *et al.*, Antigen-specific mediated suppression of β cell autoimmunity by plasmid DNA vaccination. *J Immunol*. 166:2122, 2001.
4. Seifarth, C., *et al.*, More stringent conditions of plasmid DNA vaccination are required to protect grafted versus endogenous islets in nonobese diabetic mice. *J Immunol*. (in press). 2003.

Roland Tisch, Professor in Microbiology and Immunology at the University of North Carolina. Currently, the laboratory is studying Type 1 diabetes, an autoimmune disease characterized by the T cell mediated destruction of insulin producing beta cells. The non-obese diabetic (NOD) mouse, a spontaneous animal model for Type 1 diabetes is being utilized to elucidate the key molecular and cellular events involved in the diabetogenic response.

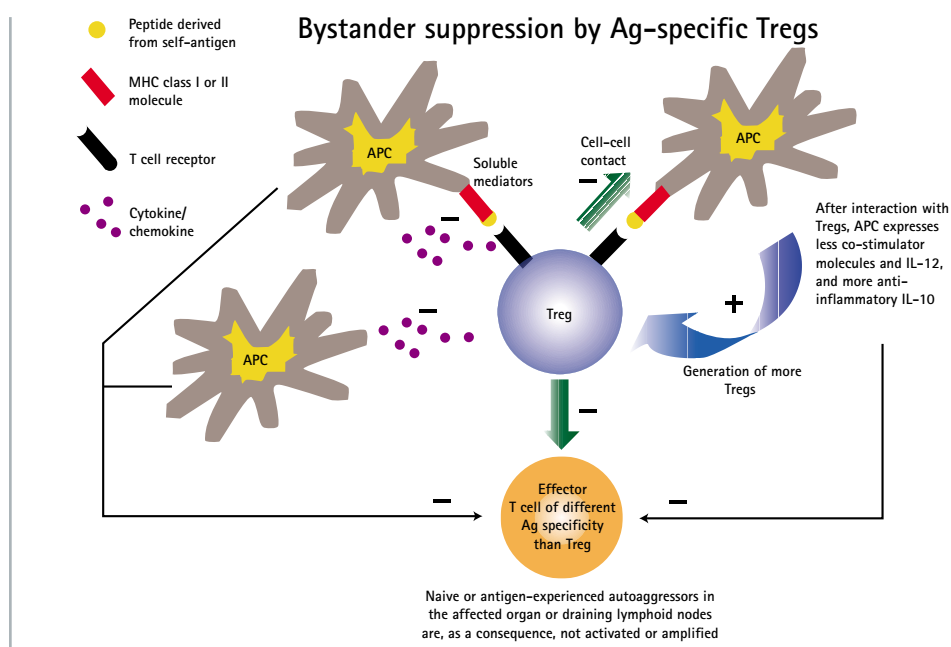
Using DNA Vaccines Expressing Islet Antigens to Prevent Type 1 Diabetes

Matthias von Herrath

Our laboratory is interested in devising novel strategies to prevent Type 1 diabetes by utilizing beta cell derived autoantigens for immunization. The underlying rationale is that self-antigens might frequently induce immune responses that are regulatory in nature. Indeed, during the past 8 years, we and other groups have identified autoreactive regulatory lymphocytes that can be induced by administering beta cell antigens, such as GAD. For example, oral administration of these antigens can induce immune deviation in the sense that islet-specific responses are skewed away from TH1 more towards a TH2 or TH3-like effector phenotype. In some cases these cells are dependent on TGF-beta, in our hands they functionally rely on secretion of interleukin-4 (IL-4). In our experimental systems, insulin-B induced IL-4 producing CD4⁺ lymphocytes can act as bystander suppressors by reducing numbers of autoaggressive CD8 T cells in the pancreatic draining lymph node

and the islets. At least in mouse models oral autoantigens require quite large dosages to be effective. Therefore, we have developed DNA vaccines that express the beta cell antigens insB and GAD. Both vaccines can prevent diabetes in the RIPLCMV or NOD mouse models. However, in order to avoid augmentation of autoaggression (which one might fear as the most undesirable outcome when immunizing with autoantigens), response modifiers in the form of cytokines and possibly anti-CD3 should be given. This strategy does not only increase the safety margin but also enhances efficacy.

We would like to propose that immunization with autoantigens such as GAD can be an effective means to prevent Type 1 diabetes, if augmentation of autoaggression is prevented by suitable response modifiers. It is noteworthy that in certain experimental situations, diabetes was prevented without response modifiers. This allows for the speculation that some islet antigen specific immune responses might be more prone to a regulatory rather than aggressive phenotype.



Matthias von Herrath, MD, Associate Member with Tenure, La Jolla Institute for Allergy and Immunology. The focus of von Herrath's research is in devising novel strategies to prevent Type 1 diabetes by inducing autoreactive regulatory T cells. Their modes of action via modulation of antigen presenting cells and combinatorial application with other immune interventions (aCD3, aCD40L) are of particular interest. Positive and negative associations between viral infections and autoimmune disease are being investigated.

Vaccination with GAD Plasmid Suppresses Diabetes in the NOD Mouse Even After Development of Insulinitis

Nora Sarvetnick

The NOD mouse develops spontaneous T cell dependent autoimmune diabetes and is used as an experimental model to explore many features shared with Type 1 diabetes patients. Much research has focused on pancreatic autoantigens and GAD65 is one of the best known targets of autoreactive T cells during the early phase of disease development. GAD expression in the pancreatic islets increases with age in the NOD mouse and when its expression is suppressed, diabetogenic T cells are not generated, resulting in protection from diabetes. The importance of GAD in Type 1 diabetes has also been confirmed by adoptive transfer experiments, that is, a GAD-specific CD4 T cell clone from a NOD mouse that normally develops diabetes transferred diabetes into NOD-scid/scid mice that normally do not develop diabetes.

Autoimmune diabetes has been successfully suppressed at the prediabetic stage via Th2 immune deviation. For example, young NOD mice injected with GAD protein (intravenously or intraperitoneally) or its peptides (intranasally) responded with a reduction of T cell proliferation to GAD65 and their insulinitis and diabetes decreased. Likewise, induction of a GAD-reactive Th2 response prolonged the survival of syngeneic islets grafted in diabetic NOD mice and inhibited disease progression in animals with early signs of Type 1 diabetes. Additionally, the transgenic expression of IL-4 in

the pancreas of NOD mice completely protected them from the disease due to the inhibition of diabetogenic Th1 lymphocytes by islet-reactive Th2 cells.

Recently it was shown that vaccination of mice with plasmids encoding foreign (viral) antigens induced effective T cell responses and circumvented the requirement for conventional immunization with proteins in complete Freund's adjuvant. We therefore hypothesized that genetic vaccination with plasmids encoding GAD may interfere with development of autoimmune diabetes. Indeed, GAD-plasmid vaccination effectively prevented diabetes in NOD female mice when they were treated at 4 to 5 weeks of age (before insulinitis) and even at 10 to 11 weeks of age (after insulinitis). This protection, however, did not stem from induction of the classical Th2 shift as reported in several other GAD-vaccination protocols. On the contrary, the mechanism of GAD-plasmid mediated diabetes protection was due to insufficient costimulation of GAD-specific T cells at the site of antigen presentation. In fact when costimulation was provided *in vivo* at the time of GAD-plasmid vaccination, the protective effect was abrogated. With these results our lab was first to report (Clinical Immunology, May, 2001) that GAD65-plasmid vaccination is effective in suppressing diabetes in the NOD mouse even after development of insulinitis and that this protection is not dependent on a systemic or pancreatic Th2 immune response.

Nora Sarvetnick is Professor of Immunology at The Scripps Research Institute (TSRI) in La Jolla. Sarvetnick is focusing on new therapies for the prevention of autoimmune diabetes.

GAD Peptides and T1DM-Associated HLA Molecules May Hold the Key to Disease Prediction and Prevention

C.B. Sanjeevi

TIDM is an autoimmune disease which occurs predominantly in children. Autoantibodies to T1DM autoantigens (GAD65 or Glutamic acid decarboxylase isoform 65, IA-2 or Tyrosine phosphatase and insulin) are used to identify ongoing autoimmune process both in newly diagnosed T1DM children and prediabetic individuals. The autoreactive T cells are implicated in the destruction of the insulin producing beta cells leading to the disease and the autoantibodies are considered only markers for the disease.

We have studied HLA-DQ that is associated with T1DM in my laboratory. These studies have helped us to identify naturally processed and presented peptides from susceptible and protective HLA-DQ, sequence these peptides and show the binding motifs (from peptide sequencing and molecular modelling).

We have screened the diabetes autoantigen GAD65 and identified several 15 amino acid long peptides that carry the right binding motifs for HLA-DQ associated with T1DM.

We believe that T cells reacting to the DQ restricted peptides from T1DM autoantigen (GAD65) are in the peripheral blood of newly

diagnosed T1DM children and prediabetic children and their detection is predictive of the disease.

An assay to identify the T cells reacting to the DQ restricted peptides has been standardized and is being tested in newly diagnosed Type 1 diabetes children and their first degree relatives. We hope that this assay will help us to both diagnose the disease and also to predict the disease in first degree relatives.

Our subsequent aim would be to use these peptides in some form of peptide-based vaccine approach.

Future potential for GAD65:

GAD65 has a very good potential in the diagnostic, predictive as well as preventive approach. The key is to find the form in which GAD65 could be used to achieve the goals. It could be whole GAD65 or modified GAD65 or peptides from GAD65.

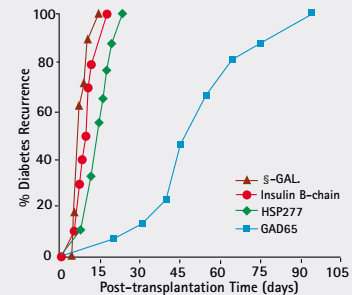


Carani B. Sanjeevi is an Associate Professor in the division of Diabetes and Endocrinology at the Department of Molecular Medicine at the Karolinska

Institute in Stockholm. Sanjeevi is also head at the 'Molecular Immunogenetics' research group at Karolinska Hospital. Sanjeevi is associated with his contribution of MHC in autoimmune Diabetes and is currently working on the role of peptides from diabetes autoantigens (GAD65) and viruses that carry motifs for diabetes associated HLA-DQ, in disease prediction.

GAD in Graphs

Immune deviation of T cell responses to GAD65 inhibits disease progression and protects islet transplants in NOD mice

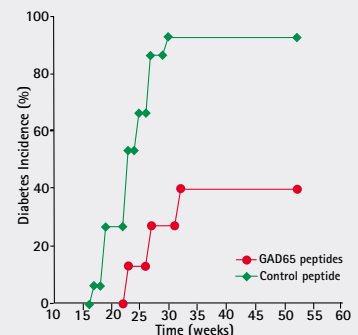


Tian *et al.* (1996) Modulating autoimmune responses to GAD inhibits disease progression and prolongs islet graft survival in diabetes-prone mice
Nature Medicine 2:1348-1353

- Pancreatic islets transplanted into diabetic NOD mice that had been pretreated with GAD65 survived longer than in those that had been pretreated with either β -galactosidase, insulin B chain or hsp65

- GAD65 treatment increases islet transplant efficacy

Nasal administration of GAD65 prevents diabetes in NOD mice



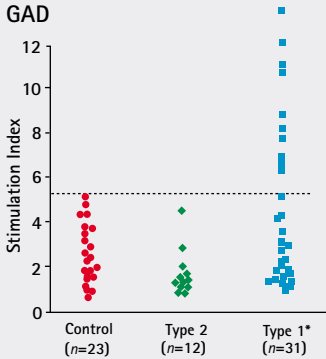
Tian *et al.* (1996) Nasal administration of glutamate decarboxylase (GAD65) peptides induces Th2 responses and prevents murine insulin-dependent diabetes
J Exp Med 183:1561-1567

- Intranasal administration of GAD65 peptide into 2-3wk old NOD mice induced IgG1 anti-GAD antibodies, reduced IFN- γ and increased IL-5 responses to GAD65 (i.e. diversion of the GAD response from Th1-Th2)

- Nasal administration of GAD65 prevents diabetes

GAD in Graphs

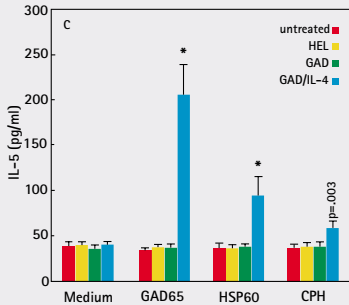
GAD ELISPOT outperforms proliferation assays for detection of GAD-reactive T cells



Kotani *et al* (2002) Detection of GAD65 reactive T cells in Type 1 diabetes by immunoglobulin-free ELISPOT assays. *Diabetes Care* 25:1390-1397

- Cellular immune responses to β -cell autoantigens were studied by proliferation and ELISPOT in PMBCs of Type 1, Type 2 diabetes patients and healthy controls
- GAD65-specific proliferation was detected in 32% Type 1 patients, while IFN- γ ELISPOT GAD65-specific activity was detected in 66% patients

Immunization with a GAD65/IL-4 plasmid DNA vaccine prevents diabetes in NOD mice



Tisch *et al.* (2001) Antigen-specific mediated suppression of cell autoimmunity by plasmid DNA vaccination. *J Immunol* 166:2122-2132

- Enhanced secretion of IL-5 and decreased secretion of IFN- γ by T cells from NOD mice immunized at 12 wks old with pDNA encoding GAD65-IgFc and IL-4 and restimulated with GAD65.
- Vaccination caused a shift in cytokine response to GAD65 stimulation

Human T Cells Recognizing GAD65 in Type 1a Diabetes

David Hafler, Jack Sadie, David Breakstone and Sally Kent

Our laboratory has been interested in the function of autoreactive T cells in humans with autoimmune disease and in particular, Multiple Sclerosis (MS) for many years. The T cell response is regulated in part through two signaling events. The first signal is through the T cell receptor by peptide processed from an antigen in the context Major Histocompatibility Complex (MHC) proteins on an antigen-presenting cell (APC) and the second is through costimulation proteins, CD28 and CTLA-4 by B7-1 and B7-2 proteins on APCs. We and others had previously found that myelin basic protein (MBP) reactive T cells in patients with MS were costimulation-independent (second signal) as compared to T cells from normal individuals. This indicates that the autoreactive T cells behaved more like T cells in a memory response and that the patient has had T cells reactive to MBP for some time. This also suggests a method for differentiating patient T cell responses from those of controls and ways of intervening in the autodestructive immune response by the T cells.

These data prompted us to examine this issue in Type 1 diabetes. Insulin-dependent Type 1a diabetes is an autoimmune disease mediated by T lymphocytes recognizing pancreatic islet cell antigens. Glutamic acid decarboxylase 65 (GAD65) appears to be an important autoantigen in the disease. We found that in patients with new-onset Type 1a diabetes, GAD65-reactive T cells were strikingly less dependent on CD28 and B7-1 costimulation to enter into cell cycle and proliferate than were equivalent cells derived from healthy controls. B7-2 appears to be the primary costimulatory molecule engaging CD28 in T cell activation of GAD65-reactive T cells, and its engage-

ment with CTLA-4 appears to deliver a negative signal. We hypothesize that these autoreactive T cells have been activated *in vivo* and have differentiated into memory cells, suggesting a pathogenic role in Type 1 diabetes. These findings strongly indicate that the activation state of antigen-specific cells plays a role in the autoimmune process and selected costimulatory molecules may represent the target of future therapies.

We are currently utilizing GAD65 for other studies in Type 1a diabetes. These include studies examining the quantitation and phenotype of GAD65 reactive T cells from controls and Type 1 diabetics with a GAD peptide-loaded tetramer (HLA DR*0401 loaded with GAD p555-567) and monitoring potentially destructive GAD65 T cell autoreactive responses in long-term Type 1a diabetics receiving islet transplants. We are enthusiastic to continue to utilize GAD65 as a means of examining autoreactive T cell responses in human Type 1a diabetes.

David Hafler, MD, is Professor of Neurology (Neuroscience) at Brigham and Women's Hospital and Harvard Medical School in Boston, MA, and the head of the Molecular Immunology Laboratory. Hafler's main clinical and research interests are in human autoimmune diseases: Multiple Sclerosis, Type 1 diabetes and rheumatoid arthritis. His goals are to understand the nature of self-recognition by T cells, to understand how that immune response leads to autoimmune disease and how one can alter this response to develop novel therapies

Sally Kent, Ph.D., is an Instructor in Neurology and Associate Immunologist at Brigham and Women's Hospital and Harvard Medical School in Boston, MA. Kent has specialized in NKT cell function in Type 1a diabetics by examining peripheral blood and pancreatic draining lymph nodes. In Type 1a diabetes, Kent has focused on GAD65 T cell reactivity as a measure of autoreactivity and memory T cell responses in controls and patients and in patients undergoing islet cell transplantation.

GAD65 Specific Regulatory T Cells May Provide Protection from Diabetes

Anthony Quinn

Numerous studies have shown that T cells of the CD8⁺ and CD4⁺ subsets are both involved in the immunopathogenesis of Type 1 diabetes (T1D) in humans. Likewise, both these cell types are also necessary for T1D in NOD mice, the murine model for spontaneous autoimmune diabetes. We have been studying the cellular immune responses to self-antigens in NOD mice to determine significance in the initiation of islet-specific damage. The focus has been on two very relevant issues regarding the initiation of autoimmune disease: the identification of host-derived factors that influence susceptibility to autoimmune disease and the role of infectious agents in the induction and persistence of autoimmunity. Although both issues are broad generalized topics – particularly the study of susceptibility factors – there are very specific issues that can be readily addressed and that provide valuable information for our understanding of the pathogenic processes in autoimmune disease.

First, does the quality of the autoimmune response to self-antigens influence the susceptibility to autoimmune disease? Recently, we have been seeking to determine if CD8⁺ T cell responses to glutamic acid decarboxylase (GAD65) are clinically relevant in the NOD mouse model of T1D. GAD65 is one of the first beta cell antigens to prime autoimmune responses that are detectable in the spleens of naive prediabetic NOD mice. Our preliminary data demonstrates that GAD65-reactive CD8⁺ T cells can be found in prediabetic NOD mice, while others have shown that such

cells are present in the peripheral blood of human patients recently diagnosed with diabetes. Currently, we are investigating the nature of GAD65-specific regulatory T cells which may provide protection from diabetes in individuals who are considered to be at-risk but remain diabetes-free. Such mechanisms may provide protection to siblings/relatives of diabetic individuals and may be represented in male NOD mice and F1 mice, both of which display a reduced susceptibility to diabetes compared to female NOD mice.

Secondly, can islet proteins, or fragments from them, be used to prevent diabetes in young NOD mice or humans? Autoimmune diseases such as diabetes are likely the result of immunological dysregulation such that the balance between the antagonistic forces of regulation and pathogenesis tilts in favor of the pathogenic mechanisms. One of our objectives is to initiate GAD65-specific mechanisms that are capable of restoring the immunological balance in diabetes-prone mice. Hopefully, the clues gained in the murine models can also provide insights into applications to human disease.

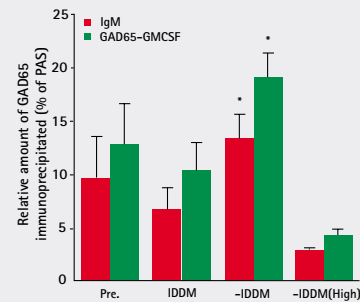


Tony Quinn, Ph.D., is Associate Professor in Biological Sciences at the University of Toledo, and Adjunct Professor in

Microbiology and Immunology at the Medical College of Ohio, Toledo, Ohio. Quinn is an immunologist with a long-standing interest in the regulation of immunity, as it relates to the control of autoimmune disease, and the enhancement of targeted immune responses. Quinn is currently studying the cellular immune responses to self-antigens in autoimmune diabetes and their roles in the initiation of pancreas-specific damage.

GAD in Graphs

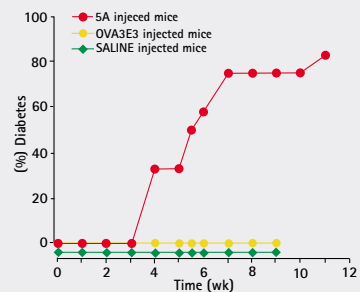
Clinical onset of IDDM is associated with a decreased Th2 GAD response



Petersen *et al* (1999). Progression to Type 1 diabetes is associated with a change in the immunoglobulin isotype profile of autoantibodies to glutamic acid decarboxylase (GAD65). Childhood Diabetes in Finland Study Group. *Clin Immunol* 90:2 276-81

- Isotype-specific IgE and IgM GAD65 autoantibodies in first degree relatives before clinical onset (Pre), IDDM at onset (IDDM), low risk IDDM relatives (-IDDM) and high risk relatives (-IDDM High).
- Individuals with a low risk for Type 1 diabetes have a higher Th2 response to GAD65 than do individuals at high risk or already with disease

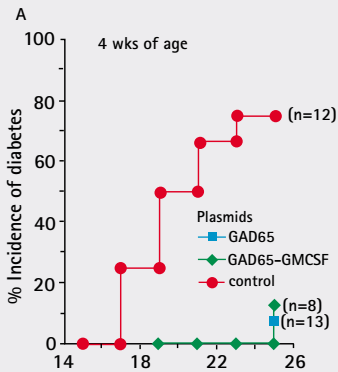
Adoptive transfer of GAD-reactive CD4⁺ Th1 cells induces diabetes in NOD/SCID mice



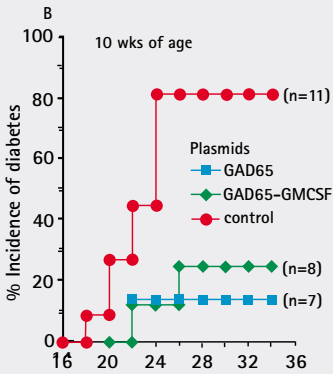
Zekzer *et al*. (1998) GAD-reactive CD4⁺ Th1 cells induce diabetes in NOD/SCID mice *J Clin Invest* 101:68-73

- Adoptive transfer of the GAD-specific CD4⁺ T cell line 5A induces diabetes in NOD/SCID mice, but transfer of an OVA-specific control T cell line (OVA3E3) does not
- GAD-specific T cells cause diabetes

GAD in Graphs



Vaccination with GAD plasmid DNA prevents diabetes in NOD mice



Balasa et al, 2001. Vaccination with GAD plasmid DNA protects mice from spontaneous autoimmune diabetes and B7/CD28 costimulation circumvents that protection
Clin Immunol 99:241-252

• Mice vaccinated at both 4-5 (A) and 10-11 weeks of age (B) were protected from developing diabetes

Is GAD All There Is?

Bart Roep

The discovery of GAD65 as one of the first islet autoantigens in the pathogenesis of type 1 diabetes was a breakthrough that enabled study of specific autoimmune responses. I worked as a postdoc in the laboratory of *Steinunn Baekkeskov* in San Francisco at the time that the identity of the 64,000 kDa target of autoantibodies was unraveled as GAD65. My aim as a T cell immunologist was to determine T cell autoreactivity to this protein in humans, and to study whether immunization of genetically predisposed mice with GAD65 led to the induction of insulinitis or diabetes. The results in these pioneer studies to check for T cell autoimmunity were unexpected since I found very few differences between patients and control subjects, although in both groups significant responses were detectable. With few exceptions, this finding turned out to be reproducible by many colleagues all over the world during the following decade. My second aim to induce diabetes by immunization with GAD65 was in vain: although very strong immune responses could be induced in various strains of mice, none of these developed insulinitis or diabetes. The latter observation proved useful for the application of using GAD65 as an immune modulator rather than as an inducer of pathogenesis. Currently there is consensus that, immunization with GAD65 delays or prevents diabetes in mice, rather than inducing or accelerating the disease.

For the next step as T cell immunologists, we

introduced GAD65 as a candidate target autoantigen in our immune monitoring of Type 1 diabetes patients receiving islet allografts. Indeed, chronic recurrent loss of islet allograft function was accompanied with increment in T cell autoreactivity to GAD65, even in the absence of increases in antibody titers, while this reactivity was never observed in patients successfully transplanted with islets. This clinical trial proved that longitudinal studies on cellular immune responses to GAD65 were informative to determine the clinical fate of beta-cells in Type 1 diabetes patients. It proved of tremendous importance to test GAD65 of high purity to avoid reactivity to contaminants in the preparation of recombinant GAD65. This was the prime result from the first international workshop on T cell reactivity to islet autoantigens. Ever since, I have used GAD65 produced and purified by *DIAMYD* as golden standard, since this material was of reproducibly high quality and void of toxic or mitogenic contaminants. A second satisfactory encounter with GAD65 came from studies in a rare autoimmune disorder called Stiff-Man Syndrome (SMS) that shares GAD65 with Type 1 diabetes as major target autoantigen. One-third of SMS patients develop Type 1 diabetes. We studied a case of SMS without Type 1 diabetes to understand why SMS patients often do not develop Type 1 diabetes despite phenomenal autoimmunity to this neuroendocrine autoantigen GAD65, in order to develop immunotherapy for Type 1 diabetes in patients that lost tolerance to this protein. Indeed, the GAD65 reactivity measured in this

“ *Indeed, chronic recurrent loss of islet allograft function was accompanied with increment in T cell autoreactivity to GAD65, even in the absence of increases in antibody titers* ”

non-diabetic SMS patient was characterized as non- or even anti-inflammatory by its IL-10 producing nature. Unexpectedly, this SMS patient did not elicit primary proliferative responses to GAD65. However, when she developed Type 1 diabetes four years later, strong primary responses were detectable, while the only cytokine produced by these T cells was the proinflammatory cytokine interferon- γ . At that time, all symptoms of SMS had resolved. This observation demonstrated that the nature of cellular autoimmunity to GAD65 is an important parameter to distinguish healthy subjects from Type 1 diabetics from SMS patients. Moreover, this was a clinical demonstration that an anti-inflammatory cytokine profile was favorable for diabetes-prone subjects against development of Type 1 diabetes.

Thirteen years after the discovery of GAD65 as target autoantigen in Type 1 diabetes mellitus through its recognition by autoantibodies, we are still left with many open issues. The disease specificity of immune responses to GAD65 is unclear. Autoantibodies against GAD65 are rarely found in non-diabetic subjects, but the majority of subjects who do have such antibodies will remain healthy, while others suffer from different autoimmune diseases. With regard to T cell responses to GAD65, the consensus is that there are only minimal differences in T cell proliferation to GAD65 in Type 1 diabetes patients versus controls. The difference most likely lies in the quality of the immune response, and how GAD65 autoimmunity is regulated. The big question now, however, is

whether islet autoantigens such as GAD65 can be used as an immunotherapeutic to divert autoimmunity from destruction to regulation.

It should also be considered to assess whether combinations of immunotherapies including GAD65 as an immune modifying agent are effective to suppress disease activity. In preliminary *in vitro* experiments we have observed that stimulation of GAD65 specific autoreactive T cell clones isolated from a prediabetic subject is affected by the new generation of humanized monoclonal antibodies against CD3, and only upon stimulation with GAD65.



Bart Roep is Associate Professor in Medicine, in particular the immunology and immunogenetics of Type 1 diabetes, at the Leiden University Medical Center, Leiden,

The Netherlands. Roep pioneered studies on the role of T cells in the pathogenesis of Type 1 diabetes, demonstrating that autoreactive T cells play a key role in beta cell destruction in humans. Roep is currently involved in the design and evaluation of new immunotherapies to prevent beta-cell autoimmunity.

Type 1 Diabetes: a Dilemma for Clinical Treatment

Mark Atkinson

Throughout much of the last decade, guarded hope existed that an agent capable of preventing or reversing Type 1 diabetes would be uncovered. As of today, such an agent does not unequivocally exist. As a result, many have addressed the question ‘why?’ The answers to this question are many; some of which are readily addressable, others are by their nature more inherently difficult. Among the latter obstacles facing the diabetes prevention field is a situation that has been referred to as the ‘treatment dilemma’. A wide body of evidence, both in animal models of Type 1 diabetes as well as in persons with or – at increased risk for – the disease, supports the notion that the most effective interventions will be those that are begun early in the autoimmune disease process. In contrast, the process of disease prediction [that is, using immunologic (e.g. GAD autoantibodies), genetic (e.g. HLA types), and metabolic (e.g. glucose tolerance tests) markers of the disease to identify risk for eventual disease development] is most accurate in the period close to the onset of overt diabetes. As a result, a conflict (both ethical and clinical) exists wherein the most effective forms of therapy may involve the early treatment of subjects in a period in which disease prediction is less accurate; a situation that has positioned the need for a safe and benign form of therapy against treating persons who may never develop Type 1 diabetes. The identification of such an idealized agent has thus far proven extremely difficult to uncover.

Yet another challenge relates to properly addressing, in combination, the questions of “who do we treat” and “what agent will we use?” In theory, attempts to prevent Type 1 diabetes will most likely address two distinct populations. The first would involve therapy of high-risk individuals (e.g. GAD/islet autoantibody positive relatives of a proband with Type 1 diabetes) or those who already

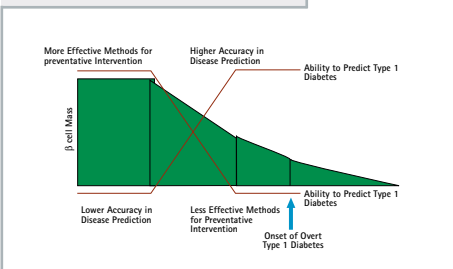
have one form of the disease (e.g. LADA subjects). The second would be that of a general population approach such as is common practice for vaccinations against infectious disease. Both models have inherent strengths and weaknesses in terms of therapeutic intervention. In the latter model, a safe and benign therapy capable of interrupting adverse immune events/environmental agents (e.g. a vaccine) or alterations in lifestyle providing avoidance of disease risk factors (e.g. diabetogenic dietary components) would ideally be implemented while the costs associated with screening general populations forms a barrier. Indeed, one could speculate that in designing preventative measures within the general population, the disease frequency and unpredictable time of onset form major obstacles that screening would be eliminated and vaccination would become universal. Performing clinical trials in increased-risk populations or those already diagnosed with the disease may prove more cost effective (in terms of a trial) and efficient, yet in terms of humanitarian benefit, it could be argued that the general population approach may ultimately be more important as approximately 85% of newly-diagnosed patients have no family history of the disease.

A final barrier for this discussion is the lack of obvious candidates for the next round of large prevention trials. As a result, current interest is directed at studies involving recent-onset Type 1 diabetes and LADA patients for the purpose of identifying new and perhaps more promising agents such as DIAMYD™. If DIAMYD™ is shown to be clinically effective, then prospective, randomized controlled studies with appropriate statistical power and objective endpoints can be designed and prevention strategies in different population groups at different stages of the disease process can be undertaken. Not only will these studies ascertain potential efficacy and safety, but should also lead to greater insight into disease pathogenesis.



Mark Atkinson, Ph.D., is an American Diabetes Association Professor for Diabetes Research, and the Director of the Center for Immunology and Transplantation at the

University of Florida. Atkinson was amongst the first groups of researchers to identify the value of measuring immune responses against GAD, and to describe the white blood cell response against this protein in persons with the disease. Atkinson holds positions on a number of scientific advisory boards/research panels including the Juvenile Diabetes Foundation International (JDFI), the American Diabetes Association and the National Institutes of Health (NIH). While Atkinson's current research extends to understanding the molecular immunological and genetic mechanisms underlying the formation of diabetes, his primary research goal lies in the development of an effective method for preventing insulin-dependent diabetes. Professors Atkinson and Noel Maclaren were first to file a US patent for GAD and diabetes therapy which is exclusively licensed by Diamyd Medical.



Diamyd S-100 β Concentration Measurement ELISA

Determination of S-100 β protein concentrations in serum is used in neurology to assess the extent of brain damage in stroke, in head injuries, during extra-corporeal circulation and during circular arrest.

It is also used for follow-up and prognosis of malignant melanoma.

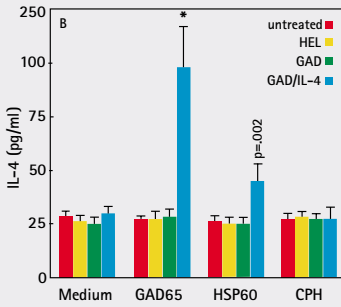
Diamyd, Inc has developed prototype ELISA kits for detecting the presence of S-100 β protein in human serum/plasma.

Diamyd S-100 β -antibody ELISA

S-100 β autoantibodies have recently been shown to be a possible marker for autoimmune diabetes. An article (Nature 2003*) shows evidence that islet cell death is related to autoreactive T- and β -cell responses to neighbouring peri-islet Schwann cells, which express S-100 β protein. Diamyd, Inc has developed prototype ELISA kits for detecting the presence of autoantibodies to S-100 β protein in human serum/plasma. The ELISA uses visual detection (requires a visible plate reader that measures absorbance at 492 nm).

GAD in Graphs

Immunization with a GAD65/IL-4 plasmid DNA vaccine prevents diabetes in NOD mice

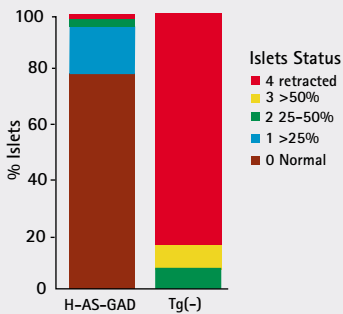


Tisch *et al.* (2001) Antigen-specific mediated suppression of cell autoimmunity by plasmid DNA vaccination. *J Immunol* 166:2122-2132

- Enhanced secretion of IL-4 and decreased secretion of IFN- γ by T cells from NOD mice immunized at 12 wks old with pDNA encoding GAD65-IgFc and IL-4 and restimulated with GAD65.

- Vaccination caused a shift in cytokine response to GAD65 stimulation

β cell-specific expression of GAD is required for development of autoimmune diabetes



Yoon *et al.* (1999) Control of autoimmune diabetes in NOD mice by GAD expression or suppression in β cells *Science* 284:1183-1190

- Islet grafts from transgenic mice expressing high levels of antisense GAD65/67 (H-AS-GAD) survive when grafted into acutely diabetic NOD mice, while grafts expressing GAD (Tg-) are infiltrated and destroyed

- GAD is the target of autoimmune attack in diabetes

Our Story of GAD – Serendipity in Science

Paul Zimmet and Ian Mackay

Our involvement with the GAD story is one of serendipity. One of us (PZ) was attending a major international pharmaceutical company advisory board meeting in Rome in 1991 and heard the presentation of one of their lead scientists, Dr. Bill Knowles. He presented work that he was involved in on islet cell antibodies. This was just around the time that GAD had been identified by Baekkeskov as the 64kD antigen that she had discovered in the 1980's – a putative key autoantigen in Type 1 diabetes. I told Bill that I was very keen to have access to a method to measure anti-GAD. Over a quiet glass of Chianti, Bill told me that he had purified GAD from pig brain and was attempting to develop an assay for antibodies but his company was not all that interested as they were more interested in Type 2 diabetes. I asked him whether I could have some GAD and he agreed. Knowles became a valued advisor and collaborator in the next phase of our work.

So the GAD came to Melbourne and within a few weeks, the nimble fingers of *Dr. Merrill Rowley* in our laboratory resulted in the development of the first radioimmunoassay (RIA) for anti-GAD, based on radioiodine labelling of the Knowles' GAD preparation. I was involved with the development of a Type 1 Diabetes Register in our island state of Tasmania so I was able to quickly find samples to test and we demonstrated high levels in newly diagnosed and long-standing cases of Type 1 diabetes. The results were published in *Diabetes* as the first RIA for anti-GAD.

Then again, serendipity came into play. We were very interested in the fact that a number of adults were identified with diabetes that presented clinically as Type 2 yet their natural

history over a period of a year or more was indicative of insulin dependency. We were aware that a close friend and colleague, *Leif Groop*, then in Finland, had undertaken a study some years previously on a group of these patients and had demonstrated a positive test for islet cell antibodies. Fortuitously, one of his outstanding young researchers, *Dr. Tiinamaija Tuomi*, had come to our laboratory as a visiting researcher. She obtained the serum samples from Groop's study and we were quickly able to measure anti-GAD in these subjects: the frequency of anti-GAD in these adult subjects was very high and appeared to be a better predictor of insulin dependency than ICA. Indeed, when vigorously challenged by a leading authority on ICA at an American Diabetes Association presentation as to our ability to perform the measurement of

ICA properly, Dr. Tuomi was able to point out that the ICA assays had been performed in his laboratory!

We then had a vigorous debate on what we should call this slow-onset Type 1 diabetes. Leif Groop favoured the term AIDA (autoimmune diabetes in adults) and PZ suggested SODA (slow onset diabetes of adults), but the wisdom and status of IM prevailed and we adopted the term LADA (latent immune diabetes in adults), not to be confused with the Mexican telephone company or the Czech motor car! Tuomi quickly wrote up the results and submitted the paper to *Diabetes* – it was accepted within 5 days. With a number of other studies with international collaborators we were able to confirm our findings in several different countries and ethnic groups. The biggest challenge still lay ahead. We were battling against the ICA “mafia” who still believed that this test was the gold standard. Would it be feasible to use the anti-GAD test to predict future diabetes?

We then came again through serendipity to a study we called “Back to the Future”. Fortuitously, my colleague in Finland, *Professor Jaakko Tuomilehto*, brought to our attention that the Finnish Women’s Register had taken blood samples from every woman during pregnancy since 1984 and these samples were stored away at the National Finnish Public Health Institute. By linking these samples to the Finnish Type 1 Diabetes Register, and testing them all for anti-GAD, we were able to show that up to 10 years prior to a woman developing Type 1 diabetes, antibodies to GAD were present. This was a critical study that confirmed the utility of the anti-GAD test as a powerful weapon for the prediction of Type 1 diabetes, and established that the “latent period” for an autoimmune disease could be very long indeed.

Later, in a landmark collaboration with the late *Professor Robert Turner* on the UKPDS cohort, we were able to demonstrate that 10% of the “pedigree” Type 2 diabetics of this cohort actually had LADA. It is now well demonstrated that this 10% figure applies in many countries around the world in terms of the number of subjects “misclassified” as having Type 2 diabetes.

Of course, early prediction of Type 1 diabetes in its long preclinical phase is not that much of

“ It is now well demonstrated that this 10% figure applies in many countries around the world in terms of the number of subjects “misclassified” as having Type 2 diabetes ”

a blessing in the absence of an effective preventive regimen. At the time of our studies on LADA referred to above, there was a peaking of interest in administration of autoantigenic preparations mucosally (oral tolerance) to abrogate autoimmune disorders diabetes included. Administration of GAD to NOD mice to retard onset of diabetes, in our studies and those of others, have given at best “promising” results. While earlier enthusiasm for oral tolerance may be diminishing, we certainly look forward to seeing results of human trials of the preventative use of GAD in LADA.

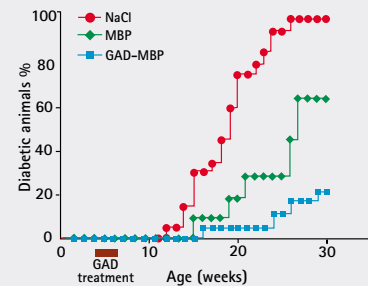


Paul Zimmet is Director of the International Diabetes Institute and Professor of Diabetes, Monash University in Melbourne, Australia.

His current research includes Type 1 diabetes etiology and the molecular mechanisms of Type 2 diabetes, insulin resistance and obesity and the effects of life-style change leading to diabetes, obesity, coronary heart disease and hypertension in developing countries in the Asia-Pacific region.

GAD in Graphs

Intraperitoneal injection of GAD65 prevents diabetes in NOD mice

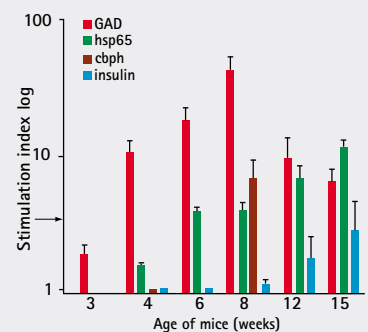


Pleau *et al.* (1995). Prevention of autoimmune diabetes in nonobese diabetic female mice by treatment with recombinant glutamic acid decarboxylase (GAD65). *Clin Immunol Pathol* 76:90-95

- 4 wk old NOD mice which had been injected ip 3 times with GAD65 were protected from diabetes development compared to mice injected with fusion protein (MBP) or saline (NaCl)

- ip administration of GAD65 prevents diabetes

GAD65 is a key target antigen in the induction of murine IDDM



Kaufman *et al.* (1993) Spontaneous loss of T cell tolerance to glutamic acid in murine insulin-dependent diabetes. *Nature* 366:69-72

- Spontaneous T cell proliferative responses in NOD mice first develop to GAD65, then to hsp65, carboxypeptidase H and to insulin in a defined order.

- GAD65 is a key diabetogenic autoantigen in murine IDDM

References

1. Kobayashi T, et al. Islet cell antibodies in Insulin-dependent and non-insulin dependent diabetics in Japan: their prevalence and clinical significance. In *clino-genetic genesis of diabetes mellitus*. Mimura G, Baba S, Goto Y, Kobberling J, Eds. Amsterdam, *Excerpta Med* 150-160 (ICS No. 597). 1982.
2. Kobayashi T, et al. Time course of islet cell antibodies and beta cell function in non-insulin-dependent stage of Type I diabetes. *Diabetes* 36: 510-7, 1987.
3. Kobayashi T, et al. Maleness as risk factor for slowly progressive IDDM. *Diabetes Care* 12: 7-11, 1989.
4. Kobayashi T. Subtype of insulin-dependent diabetes mellitus (IDDM) in Japan: slowly progressive IDDM-the clinical characteristics and pathogenesis of the syndrome. *Diabetes Res Clin Pract* 24 Suppl: S95-9. Review, 1994.
5. Kobayashi T, et al. GAD antibodies seldom disappear in slowly progressive IDDM. *Diabetes Care* 19: 1031, 1996.
6. Kobayashi T, et al. Immunogenetic and clinical characterization of slowly progressive IDDM. *Diabetes Care* 16: 780-8, 1993.
7. Nakanishi K, et al. Predictive value of insulin autoantibodies for further progression of beta cell dysfunction in non-insulin-dependent diabetics. *Diabetes Res* 9: 105-109, 1988.
8. Nakanishi K, et al. Exocrine pancreatic ductograms in insulin-dependent diabetes mellitus. *Am J Gastroenterol* 89: 762-6, 1994.
9. Nakanishi K, et al. Relationships among residual beta cells, exocrine pancreas, and islet cell antibodies in insulin-dependent diabetes mellitus. *Metabolism* 142: 196-203, 1993.
10. Groop LC, et al. Islet cell antibodies identify latent Type I diabetes in patients aged 35-75 years at diagnosis. *Diabetes* 35: 237-41, 1986.
11. Zimmet PZ, et al. Latent autoimmune diabetes mellitus in adults (LADA): the role of antibodies to glutamic acid decarboxylase in diagnosis and prediction of insulin dependency. *Diabet Med* 11: 299-303, 1994.
12. Thai AC, et al. Anti-GAD antibodies in Chinese patients with youth and adult-onset IDDM and NIDDM. *Diabetologia* 40: 1425-1430, 1997.
13. Sutaneegara D, et al. The epidemiology and management of diabetes mellitus in Indonesia. *Diabetes Res Clin Pract* 50 Suppl 2: S9-S16, 2000.
14. Brooks-Worrell BM, et al. Cellular immune responses to human islet proteins in antibody-positive Type 2 diabetic patients. *Diabetes* 48: 983-8, 1999.
15. Kobayashi T, et al. Small doses of subcutaneous insulin as a strategy for preventing slowly progressive beta cell failure in islet cell antibody-positive patients with clinical features of NIDDM. *Diabetes* 45: 622-6, 1996.
16. Nakanishi K, et al. Residual beta cell function and HLA-A24 in IDDM. Markers of glycemic control and subsequent development of diabetic retinopathy. *Diabetes* 44: 1334-9, 1995.
17. Kobayashi T, et al. Insulin Intervention to Preserve β -cells in Slowly Progressive Insulin-Dependent (Type 1) Diabetes Mellitus. *Ann NY Acad Sci* 958: 117-30, 2002.

Tetsuro Kobayashi, MD, Ph.D., Kanazawa University School of Medicine, Kanazawa, Professor and Chairman, Third Department of Internal Medicine, School of Medicine, University of Yamanashi, Tamaho City, Yamanashi, 409-3898 Japan 1968-1974. Chief, Department of Endocrinology and Metabolism, Toranomon Hospital and Okinaka Memorial Institute of Medical Research, Tokyo. Main Works: Discovery of the presence of SPIDDM, Insulin intervention of SPIDDM.

Intervention to Preserve β -Cells in SPIDDM

Tetsuro Kobayashi, Shoichiro Tanakaa, Kaoru Aidaa and Taro Maruyamab

Since the late 1970's our laboratory has made rigorous efforts to examine the clinical significance of the presence of islet cell antibodies (ICA) in patients with non-insulin-dependent diabetes mellitus (NIDDM) (1-6). We have found that the clinical features of ICA-positive NIDDM patients are largely different from ICA-negative NIDDM patients because β -cell dysfunction is progressive and most of them lapse into an insulin dependent state indistinguishable from that of insulin-dependent diabetes mellitus (IDDM) (2-6). In 1982, we described ICA-positive NIDDM as slowly progressive insulin-dependent diabetes mellitus (SPIDDM) based on the characteristic clinical courses (1). The clinical characteristics of SPIDDM include; 1) Late age onset with an initial clinical phenotype of NIDDM with progressive β -cell failure and subsequent features of IDDM (1-4); 2) Persistent pancreatic humoral autoimmune markers including glutamic acid decarboxylase autoantibody (GADAb), ICA, ICA512/IA-2 autoantibodies (IA-2Ab) and insulin autoantibodies (IAA) (2, 5, 7); 3) Male predominance (3, 6); 4) Involvement of exocrine as well as endocrine pancreas (8); 5) Less marked insulinitis with preserved β -cell mass (9); 6) Association with specific HLA-DQ A1*0302-DQB1*0401 haplotype (6). After development of a convenient GADAb assay, an increasing number of studies have shown that NIDDM patients who have GADAb as well as ICA are confirmed to be distinct from GADAb-negative NIDDM patients and are called latent Type 1 diabetes (10) or latent autoimmune diabetes in adults (LADA) (11).

The clinical importance for intervention to maintain β -cell function or to prevent β -cell failure in SPIDDM is based on the following points: 1) SPIDDM is more prevalent than classical IDDM and the prevalence is as high as 10% among NIDDM patients in some ethnic groups including Caucasian (10, 11), Japanese (5), Chinese (12), Indonesian (13), and Thai (4) populations. 2) the T cell response to islet antigens in SPIDDM is weaker

than that in classical IDDM (14). 3) A pilot study demonstrated that small doses of insulin prevent β -cell failure in SPIDDM patients (15). 4) Insulin secreted from preserved β -cells in SPIDDM contributes to stable glycemic control and subsequently prevents late diabetic complications (16). In 1996, we organized a multicenter randomized clinical trial (The Tokyo Study) to examine the effect of early treatment with insulin in SPIDDM. At seven hospitals in the Tokyo area, about 4000 NIDDM patients were screened for autoantibodies against GAD. Patients were randomly assigned to one of two groups. One group received subcutaneous insulin injection (Insulin group) and the other received oral sulfonylurea (SU group). The primary outcome measures for the study were serum C-peptide response and level of blood glucose during 75g OGTT.

During the trial C-peptide responses to OGTT (Sigma C-peptide) decreased progressively in the SU group and became significant at 24 and 36 months (17). Seven patients lapsed into an insulin-dependent stage when their sigma C-peptide reached less than 4 ng/ml. In contrast, the sigma C-peptide value remained unchanged in the patients in the Insulin group and the value was significantly different from that of the SU group at 36 months (17).

Our multicenter randomized study demonstrated that insulin intervention is effective and safe for gradual β -cell failure in SPIDDM, specifically in the patients with preserved β -cell function and high titer of GADAb at the initiation of insulin.

In a recent study, we have found GADAbs to a unique epitope in the N-terminal region of the GAD65 molecule. This region includes anchoring domains of GAD65 molecules, which potentially can be accessed by GADAb during the exocytosis of GABA from the β -cell (unpublished data). These results open the door to the prevention of β -cell failure by vaccination of GAD in SPIDDM patients, because GADAb may have a causative role in β -cell dysfunction and vaccination with GAD may modify the pathological process of β -cell failure in this syndrome.

GAD Antibodies and Latent Autoimmune Diabetes of the Adult (LADA)

A.G. Unnikrishnan and S. K. Singh

The prevalence of Type 2 diabetes is rapidly increasing in the Indian subcontinent. While the majority of subjects with Type 2 diabetes in developed countries are obese, those from India are mostly non-obese, and many of them are lean (1). The proposed hypotheses to explain this include coexistent malnutrition, autoimmunity and metabolic abnormalities.

Recent research from North India shows that one-fourth of the recently diagnosed Type 2 diabetics who are lean (i.e. with a body mass index of less than 18.5 kg/m²) have positivity to GAD antibodies. These adult subjects with autoimmunity were significantly younger, had a lower waist hip ratio and beta cell function (HOMA) as compared to lean Type 2 diabetics without antibody positivity. Hence they had a clinical profile consistent with latent autoimmune diabetes of the adult (LADA). They also had lower insulin resistance (HOMA), showing that reduced beta cell function is the predominant metabolic abnormality in these subjects (2).

The above study included only adult-onset Type 2 diabetes, which is seen commonly in India. There is also evidence that 23% of emaciated, very young subjects with a ketosis-resistant form of diabetes, a phenotype which is rare yet peculiar to India, are positive for GAD antibodies (3). This form of diabetes has also been termed malnutrition modulated diabetes mellitus (MMDM).

Exciting work is in progress to unravel the genetic basis of Indian diabetics with GAD65 antibody positivity. It has been reported that MHC-related genes can discriminate between acute-onset and slow-onset Type 1 diabetes in India, and can also distinguish the MMDM phenotype (4).

Clearly, it is important to avoid the misclassification of these lean diabetic subjects, as some of them could have LADA. The detection of GAD antibodies could predict the early onset of insulin dependency, prompting more aggressive glucose-lowering therapy. This could prevent unnecessary exposure to hyperglycemia. Further Indian studies are needed to assess the prevalence of eventual beta cell failure in these subjects, and the speed of progression.

References

1. Mohan V, *et al*. Clinical Profile of lean NIDDM in South India. *Diabetes Res Clin Pract* 38(2): 101-8, 1997.
2. Unnikrishnan AG, *et al*. Clinical and immunological profile of underweight Type 2 diabetes in north India. [Abstract number: A-03-255-EASD]. Accepted for presentation at the 18th International Diabetes Federation Congress, 24 - 29 August 2003, Paris, France.
3. Singh AK, *et al*. Role of islet autoimmunity in the aetiology of different clinical subtypes of diabetes mellitus in young north Indians. *Diabet Med* Apr;17(4):275-80, 2000.
4. Sanjeevi CB, *et al*. MHC class I chain-related gene alleles distinguish malnutrition-modulated diabetes, insulin-dependent diabetes, and non-insulin-dependent diabetes mellitus patients from eastern India. *Ann N Y Acad Sci* Apr 958:341-4, 2002.

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LADA Diagnostics and GAD Transgenic Plants

Alberto Falorni

My main research interest is pathogenesis and prevention of Type 1 diabetes mellitus and other endocrine autoimmune diseases. I have initially focused my attention on the role of glutamic acid decarboxylase (GAD65) as a target molecule of autoantibodies in Type 1 diabetes. The development of a semi-automated procedure for the radioimmunological determination of GAD65 autoantibodies in human serum (1) has made it possible to test the diagnostic sensitivity (frequency of Type 1 diabetic patients positive) and specificity (frequency of non-diabetic subjects negative) of this immune marker for the disease. It was shown that GAD65 autoantibodies can be detected in over 80% of recently diagnosed Type 1 diabetic patients and occur more frequently among diabetic females than males. Interestingly, GAD65Ab have emerged as the immune marker at highest diagnostic sensitivity for adult-onset Type 1 diabetes (2) which has paved the way to the diagnostic use of this marker in routine clinical practice to discriminate autoimmune from non-autoimmune cases.

In the attempt of both identifying novel markers at highest diagnostic accuracy for Type 1 diabetes and elucidating the molecular mechanisms of autoantibody formation, I have constructed chimeric molecules, generated by substitution of regions of human GAD65 with homologous regions of GAD67 (a

COOH-terminal end of the autoantigen discriminate Type 1 diabetic children from antibody-positive children who do not progress towards clinical diabetes. In addition, I have constructed a mutant form of human GAD65, generated by site-directed mutagenesis of the active site of the enzyme, which has proven enzymatically inactive but immunologically indistinguishable from "wild-type" human GAD65.

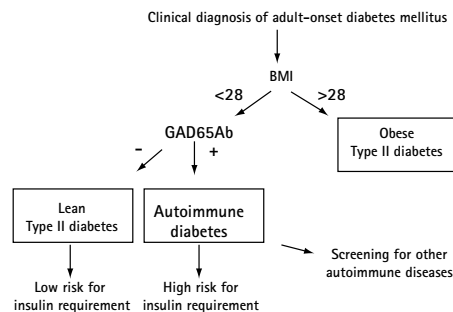
More recently, I have focused my interest on the diagnosis and clinical characteristics of the so-called latent autoimmune diabetes in adults (LADA). I have demonstrated that GAD65 antibodies can be detected in approximately 10% of a hospital-based population of patients diagnosed with Type 2 diabetes on clinical grounds in Italy (4). Among GAD65Ab-positive individuals, high antibody levels and presence of antibodies directed to the COOH-terminal end of the autoantigen predicted an extremely high risk of progression towards insulin-dependency and of associated organ-specific autoimmune diseases, such as thyroid autoimmune diseases or autoimmune Addison's disease. In contrast, the presence of low levels of GAD65 antibodies directed only against the middle region of the enzyme discriminated a sub-population of LADA patients with clinical characteristics very similar to that of antibody-negative Type 2 patients.

At present, I am testing the hypothesis that the islet autoimmune process may be modulated and the appearance of clinical signs of the disease delayed or prevented by the oral administration of recombinant human GAD65. To make this strategy potentially applicable to clinical studies of primary prevention, we have constructed transgenic plants expressing human GAD65 (5), that can potentially allow us to administer the human autoantigen without the need for costly and time-consuming procedures of protein purification. We have demonstrated that, in transgenic plants, GAD65 accumulates in chloroplast thylakoids and mitochondria and that the targeting of human GAD65 to the plant cell cytosol (by substitution of the NH₂-terminal end of the protein with a homologous region of GAD67) is associated with a 5-fold increase of expression levels. Ongoing studies are testing the effect of the oral administration of transgenic plant material containing human GAD65 in animal models of spontaneous autoimmune diabetes.

References

1. A.Falorni, *et al.*, Radioimmunoassays for glutamic acid decarboxylase (GAD65) and GAD65 autoantibodies using 35S or 3H recombinant human ligands. *J Immunol. Methods* 186:89-99,1995
2. C.L.Vandewalle, *et al.*, Belgian Diabetes Registry: High diagnostic sensitivity of glutamate decarboxylase autoantibodies in IDDM with clinical onset between age 20 and 40 years. *J Clin. Endocrinol. & Metab.* 80:846-851,1995
3. A.Falorni, *et al.*, Diagnostic sensitivity of immunodominant epitopes of glutamic acid decarboxylase (GAD65) autoantibodies in childhood IDDM. *Diabetologia* 39:1091-98,1996
4. A.Falorni, *et al.*, Autoantibody recognition of COOH-terminal epitopes of GAD65 marks the risk for insulin requirement in adult-onset diabetes mellitus. *J Clin. Endocrinol. Metab* 85:309-316,2000.
5. L.Avesani, *et al.*, Improved in planta expression of the human islet autoantigen glutamic acid decarboxylase (GAD65). *Transgenic Research* 12:203-212,2003
6. Marcovina *et al.*, Evaluation of a novel radioimmunoassay using ¹²⁵I-labelled human recombinant GAD65 for the determination of glutamic acid decarboxylase (GAD65) autoantibodies. *Int J Lab Res* (2000) 30: 21-26

Alberto Falorni, MD, PhD is Associate Professor in Internal Medicine at the University of Perugia, Italy. Falorni has specialized in the techniques for the study of humoral autoimmunity and genetics of Type 1 diabetes mellitus and other endocrine autoimmune diseases. Falorni's clinical activity is focused on the diagnosis and treatment of endocrine diseases. Falorni's main scientific interests are pathogenesis and prevention of Type 1 diabetes mellitus and of autoimmune primary adrenal insufficiency.



GAD isoenzyme which is not a major diabetes-related autoantigen), to define the epitope regions of the autoantigen recognized by human autoantibodies (3). It was shown that human GAD65 autoantibodies are primarily directed against epitopes located in the middle and COOH-terminal regions of the enzyme and that levels of GAD65 antibodies specific for the

T1DM and LADA Differ in GADA Epitope Specificity

Christiane Hampe, Rattan Juneja, Åke Lernmark, Jerry Palmer

Diabetes Mellitus is classified into two major forms, Type 1 and Type 2 diabetes. Type 1 diabetes is characterized by an autoimmune-mediated destruction of beta-cells, leading to insulin deficiency. The autoimmune reaction involves both T cells and antibodies directed against islet cell autoantigens that can be detected in the majority of Type 1 diabetes patients (1, 2). The main autoantigens identified are insulin (3), the Mr 65,000 isoform of glutamic acid decarboxylase (GAD65) (4), and the tyrosine phosphatase-like IA-2 antigen (5). These autoantibodies are often detected long before the clinical onset of Type 1 diabetes and are useful to predict disease risk (5, 6). GAD65 and IA-2 autoantibodies (Ab) are readily detected by now standardized (7), precise and reproducible radioimmunoassays (4, 7, 8) suitable for large scale analysis and population screening (6, 9). In contrast, classical Type 2 diabetes patients do not show evidence of autoimmune beta cell destruction. Patients with Type 1 diabetes usually require insulin treatment at the time of diagnosis whereas Type 2 patients can be successfully treated by diet and oral agents for many years. These patients do not show evidence of autoimmune beta cell destruction. A third group of patients is referred to as latent autoimmune diabetes in adults (LADA) (10), Type 1.5 diabetes (11), or slowly progressive insulin dependent diabetes mellitus (SPIDDM) (12). These patients lose beta cell function, fail oral agents early and require insulin treatment (13, 14). Evidence for an underlying autoimmune pathogenesis is provided by the observation that many of these patients have islet cell antibodies (ICA), autoantibodies to GAD65 (GAD65Ab) (10, 15, 16), or both. The presence of GAD65Ab alone is a sufficient marker for future insulin requirement in younger patients (44 years or younger) while in older patients positivity for both ICA and GAD65Ab is a stronger predictor of insulin require-

ment (17). The question has been raised whether Type 1.5 diabetes represents a separate clinical disease or is a slowly progressive form of Type 1 diabetes (18, 19). Epitope mapping of GAD65Ab can assist in the classification of the underlying autoimmunity. Using both GAD65/67 fusion proteins (20, 21) and GAD65-specific recombinant Fab we and others were able to identify phenotype-specific GAD65Ab epitopes. GAD65Ab in newly diagnosed young Type 1 diabetes patients recognize restricted epitopes primarily located at the combined middle-carboxyterminal conformational epitope of GAD65, while binding to GAD67 or the N-terminus of GAD65 is detected only at a low level. In contrast, GAD65Ab positive Type 1.5 diabetes patients exhibit a GAD65Ab epitope pattern that is characterized by binding to both the N-terminus of GAD65 and to a tentative conformational epitope formed of the middle and carboxyterminal part of GAD65 (22). This GAD65Ab epitope profile clearly differs from that found in Type 1 diabetes patients and more resembles the broader GAD65Ab epitope specificity found in GAD65Ab-positive healthy individuals and first-degree relatives (20). This difference in the binding pattern of GAD65Ab of Type 1.5 diabetes patients compared to that of Type 1 diabetes patients supports the notion that the disease process may differ between these two types of patients. We therefore suggest that Type 1.5 diabetes might be a subtype of Type 1 diabetes characterized by separate immunologic features. GAD65Ab epitope patterns may be useful to identify Type 1.5.

Christiane Hampe, Ph.D., has a position as junior faculty at the University of Washington in Seattle. Her research interests are the dissection of the role of GAD65 and its autoantibodies in the pathogenesis of Type 1 diabetes. While these autoantibodies are widely accepted as markers for the disease, preliminary data indicate that disease-specific GAD65Ab modulate T cell responses. Hampe's current research goals are to understand the effect of GAD65Ab on processing and presentation of GAD65.

References

1. Bonifacio E, et al. Islet autoantibody markers in IDDM: risk assessment strategies yielding high sensitivity. *Diabetologia* 38:816-822, 1995
2. Landin-Olsson M, et al. Islet cell and other organ-specific autoantibodies in all children developing Type 1 (insulin-independent) diabetes mellitus in Sweden during one year and in matched controls. *Diabetologia* 32:387-395, 1989
3. Palmer JP, et al. Insulin antibodies in insulin-dependent diabetics before insulin treatment. *Science* 222:1337-1339, 1983
4. Grubin CE, et al. A novel radioligand binding assay to determine diagnostic accuracy of isoform-specific glutamic acid decarboxylase antibodies in childhood IDDM. *Diabetologia* 37:344-350, 1994
5. Verge CF, et al. Prediction of Type 1 diabetes in first-degree relatives using a combination. *Diabetes* 46:1701-1710, 1997
6. Bingley PJ, et al. Prediction of IDDM in the general population: Strategies based on combinations of autoantibody markers. *Diabetes* 46:1701-1710, 1997
7. Mire-Sluis AR, et al. The development of a World Health Organisation international standard for islet cell antibodies: the aims and design of an international collaborative study. *Diabetes Metab Res Rev* 15:72-77, 1999
8. Verge CF, et al. Combined use of autoantibodies (IA-2 autoantibody, GAD autoantibody, insulin autoantibody, cytoplasmic islet cell antibodies) in Type 1 diabetes: Combinatorial Islet Autoantibody Workshop. *Diabetes* 47:1857-1866, 1998
9. Rolandsson O, et al. Glutamate decarboxylase (GAD65) and tyrosine phosphatase-like protein (IA-2) autoantibodies index in a regional population is related to glucose intolerance and body mass index. *Diabetologia* 42:555-559, 1999
10. Tuomi T, et al. Antibodies to glutamic acid decarboxylase reveal latent autoimmune diabetes mellitus in adults with a non-insulin-dependent onset of disease. *Diabetes* 42:359-362, 1993
11. Harris ML, et al. Classification of diabetes mellitus and other categories of glucose intolerance. In: Keen H, DeFronzo R, Alberti K, Zimmet P, ed. *The international textbook of diabetes mellitus*. London: Wiley, 1992, 3-18.
12. Ludvigsson J, et al. HLA-DR3 is associated with amore slowly progressive form of Type 1 (insulin-dependent) diabetes. *Diabetologia* 29:207-210, 1986
13. Temple RC, et al. Insulin deficiency in non-insulin-dependent diabetes. *Lancet* 1:293-295, 1989
14. Gjesing HJ, et al. Fasting plasma c-peptide, glucagon stimulated plasma c-peptide, and urinary c-peptide in relation to clinical type of diabetes. *Diabetologia* 32:305-311, 1989
15. Groop LC, et al. Islet cell antibodies identify latent Type 1 diabetes in patients aged 35-75 years at diagnosis. *Diabetes* 35:237-241, 1986
16. Rowley MJ, et al. Antibodies to glutamic acid decarboxylase discriminate major types of diabetes mellitus. *Diabetes* 41:548-551, 1992
17. Turner R, et al. UKPDS 25: autoantibodies to islet T cell cytoplasm and glutamic acid decarboxylase for prediction of insulin requirement in Type 2 diabetes. *UK Prospective Diabetes Study Group [published erratum appears in Lancet] 1998 Jan 31;351 (9099): 3761*. *Lancet* 350:1288-1293, 1997
18. Juneja R, et al. Autoimmunity 29:65-83, 1999
19. Tuomi T, et al. Clinical and genetic characteristics of Type 2 diabetes with and without GAD antibodies. *Diabetes* 48:150-157, 1999
20. Hampe CS, et al. Recognition of Glutamic Acid Decarboxylase (GAD) by Autoantibodies from Different GAD Antibody-Positive Phenotypes. *J Clin Endocrinol Metab* 85:4671-4679, 2000
21. Falorni A, et al. Diagnostic sensitivity of immunodominant epitopes of glutamic acid decarboxylase (GAD65) autoantibodies epitopes in childhood IDDM. *Diabetologia* 39:1091-1098, 1996
22. Hampe CS, et al. GAD65 antibody epitope patterns of Type 1.5 diabetic patients are consistent with slow-onset autoimmune diabetes. *Diabetes Care* 25:1481-1482, 2002

GAD and Batten's Disease

David Pearce

The discovery that individuals with juvenile neuronal ceroid lipofuscinosis, or Batten disease have circulating autoantibodies to GAD65 is perhaps the most recent chapter on GAD65 autoantibodies and disease, and certainly one that requires further exploration. We first published that individuals with Batten disease, and a mouse model for the disease had circulating autoantibodies to GAD65 in *Human Molecular Genetics* in 2002 (1). The circumstances that led to this report do not necessarily follow conventional scientific reasoning, as much as to intuition.

Batten disease is a pediatric onset devastating fatal neurodegenerative disease. Individuals with Batten disease have inherited mutations in both copies of a still to be functionally characterized gene product, designated CLN3 (2). We were studying a mouse model that lacks a CLN3-gene product constructed by others (3). Gene expression studies indicated that in the brain of cln3-knockout mice, a shift in the expression of enzymes associated to the synthesis and utilization of the neurotransmitter glutamate was altered. We next confirmed that endogenous levels of glutamate/glutamic acid were elevated in the brains of cln3-knockout mice. There was no reason to suspect, and still isn't, that the CLN3-gene product is directly involved in glutamate metabolism. I convinced *Subrata Chattopadhyay* the postdoctoral fellow working with me on this to use serum drawn from the cln3-knockout mice as the primary antibody in a western blot against a brain protein extract. The basis for this was that a block in GAD activity, which converts glutamate to GABA could be mediated by the presence of an autoantibody to GAD, thus causing the elevation in glutamate that we observed. It turned out that the cln3-knockout mice sera had circulating autoantibodies reactive to a number of brain proteins. Most notably, there was a predominant protein of around 65kD. We knew that this would be GAD65, we just had to prove it. Thanks to the vast amount of research on GAD65 in diabetes and stiff persons syndrome we were able to benefit from reagents available and a plethora of published techniques. Within the space of a few

months Subrata confirmed that the cln3-knockout mice in addition to having elevated glutamate in the brain, also had a decrease in enzymatic activity of GAD, most likely due to a demonstrated association of autoantibody in the brain. His *in vitro* studies showed that serum drawn from the cln3-knockout mice also contained an element that could inhibit the activity of GAD, and that we could block the reactivity of the autoantibody to GAD65 by pre-incubation with recombinant GAD. At this point I felt we had biochemically met the criteria of showing that the autoantibody could block an enzyme, GAD, resulting in accumulation of its substrate, glutamate, and that association to the brain resulted in a decrease in the activity of the enzyme. The final piece of this initial study was to show a possible link between the autoantibody and the disease. I might add that this in many ways is an ongoing task.

Nevertheless, this is where *Jim Powers*, a neuropathologist came to the rescue. With the aid of his postdoctoral fellow, *Masumi Ito*, he confirmed a decrease in GAD65 positive neurons in Batten disease post-mortem brain. I might add that Jim did a huge amount of other work that aided our understanding of this phenomenon, and most importantly, he believed what initially was a strange idea, namely that GAD65 autoantibodies may be present in the disease. Of course we have gone on to show that individuals with Batten disease have autoantibodies to GAD65, in fact to date every child we have tested has come up positive. Furthermore, we have shown that other pediatric neurodegenerative diseases that fall in a similar category to Batten disease do not have circulating autoantibodies to GAD65 (4). Our focus right now is to deduce whether GAD65 autoantibodies are really pathological, or simply epi-phenomenal in Batten disease. This of course is a mammoth biological task, however, thanks again to the resources already put forth in researching GAD65 autoantibodies in other diseases, and some excellent collaborations with researchers in this field, I believe we will again benefit in expediting answering this question. Importantly, establishing the potential contribution of the autoantibody to Batten disease, will reveal whether or not this is an element of the disease process that should be targeted.

References

1. Chattopadhyay, S, *et al*. An autoantibody inhibitory to glutamic acid decarboxylase in the neurodegenerative disorder Batten disease. *Hum. Mol. Genet.* 11, 1421-1431, 2002.
2. International Batten Disease Consortium. Isolation of a novel gene underlying Batten disease. *Cell* 82, 949-957, 1995.
3. Mitchison, H. M., *et al*. Targeted disruption of the Cln3 gene provides a mouse model for Batten disease. *Neurobiol. Dis.* 6, 321-334, 1999.
4. Chattopadhyay, S, *et al*. An autoantibody to GAD65 in sera of patients with juvenile neuronal ceroid lipofuscinoses. *Neurology.* 59, 1816-1817, 2002.

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GAD and Parkinson's Disease

Helen Fitzsimons and Matthew During

Our primary interest in GAD has been in the development of gene therapy based treatments for neurological disorders that involve abnormalities in neuronal activity. As neurotransmission in the brain can be modulated by manipulation of GABA levels, we are using adeno-associated viral vector (AAV)-mediated gene transfer of GAD into targeted brain areas to augment inhibition. The expression of GAD in neurons, which contain high intracellular levels of glutamate and co-factors, leads to biosynthesis and release of GABA, which acting on GABA_A receptors facilitates transport of chloride ions into the cell, hyperpolarization and relevant silencing of cell activity. AAV is our preferred gene delivery vector as it provides a high level of stable gene expression with minimal inflammatory or immune response (Xu *et al*, 2001; Mastakov *et al*, 2002) and in addition, direct injection of AAV allows transduction of specific neuronal pathways without affecting the rest of the brain (Xu *et al*, 2001; During *et al*, 2003).

Our main focus to date has been on the development of an AAVGAD gene therapy based treatment for Parkinson's disease (PD). The motor abnormalities of PD are caused by alterations in basal ganglia network activity. Briefly, the death of dopaminergic neurons in the substantia nigra pars compacta and the associated depletion of dopamine in the striatum causes disinhibition of the subthalamic nucleus (STN). This in turn causes overactivation of the output nuclei of the basal ganglia, the substantia nigra pars reticulata (SNr) and the internal segment of the globus pallidus, leading to impaired motor function.

We rationalized that an increase in GABAergic transmission from the STN to the SNr would lead to suppression of firing activity of SNr neurons. AAVGAD65 or AAVGAD67 gene transfer to the rat STN via stereotactic surgery resulted in robust expression of recombinant GAD protein that was restricted to STN neurons, and a four-fold increase in GABA release from the SNr following stimulation of the STN. Single unit recording from the SNr following stimulation of the STN showed rare (6%) inhibitory responses in

control rats, which was increased to 78% in GAD65-treated rats ($P < 0.001$) and 33% in GAD67-treated rats ($P < 0.02$) (Luo *et al*, 2002).

To model Parkinson's Disease by degeneration of the nigro-striatal dopaminergic pathway in the rat, 6-hydroxydopamine-induced lesioning of the medial forebrain bundle results in apomorphine-induced rotational behaviour contralateral to the denervated side. Rats that were lesioned three weeks following GAD65 gene transfer showed a 65% decrease in rotation rate compared to control rats and 35% and 80% survival of dopaminergic neurons in the SNc and ventral tegmental area, respectively (measured by tyrosine hydroxylase immunoreactivity) compared to a 93-99% loss of these dopaminergic neurons in control rats (Luo *et al*, 2002).

These data showed that transfer of GAD to STN neurons could shift predominantly excitatory responses to inhibitory ones, resulting in neuroprotection and an improvement in motor function. Toxicology studies did not reveal the presence of any inflammatory or immune responses in rats or monkeys. Further data from primate studies is currently being analyzed and a phase I clinical trial for AAVGAD gene transfer to human Parkinson's disease patients has been approved by the FDA (During *et al*, 2001).

We also have an interest in the development of gene therapy treatments for temporal lobe epilepsy and have studies underway to examine whether AAV-mediated transfer of GAD65 to the rat hippocampus can decrease the excessive electrical output of the perforant pathway that occurs during seizures. Overexpression of AAVGAD65 in the rat hilus led to a decrease in kainic-acid induced seizures and an associated decrease in hippocampal neurodegeneration (unpublished data).

In summary, we believe the use of GAD gene transfer holds potential for treatment of PD and other neurological disorders associated with excessive excitation.

References

1. Luo J, *et al*, During MJ Subthalamic GAD gene therapy in a Parkinson's disease rat model. *Science* 298, 311-482, 2002.
2. During MJ, *et al*, Development and optimization of adeno-associated virus vector transfer into the central nervous system. *Methods Mol Med*. 76:221-36, 2003.
3. Xu R, *et al*, Quantitative comparison of expression with adeno-associated virus (AAV-2) brain-specific gene cassettes. *Gene Ther*. 8, 1323-32, 2001.
4. During MJ, *et al*, Subthalamic GAD gene transfer in Parkinson disease patients who are candidates for deep brain stimulation. *Hum Gene Ther*. 12, 1589-91, 2001.
5. Mastakov MY, *et al*, Immunological aspects of recombinant adeno-associated virus delivery to the mammalian brain. *J Virol*. 76, 8446-54, 2002.

Matthew During is Professor of Molecular Medicine in the Department of Molecular Medicine & Pathology at the University of Auckland, Auckland, New Zealand. During's interests include the development and characterization of novel therapeutics for neurological disorders. Specifically, During together with his Ph.D. student, Helen Fitzsimons have been exploring the use of GAD as a therapeutic gene for Parkinson's Disease and Epilepsy.

Diamyd's Commercial Development of a GAD Vaccine

John Robertson

Diamyd Medical has been pioneering both the evaluation of clinical safety and clinical efficacy of GAD – with a view to its potential use as a vaccine to prevent autoimmune diabetes. Both these achievements were made possible by Diamyd's early definition of a manufacturing process – capable of providing the quantity and quality of GAD required for different stages of commercial drug development.

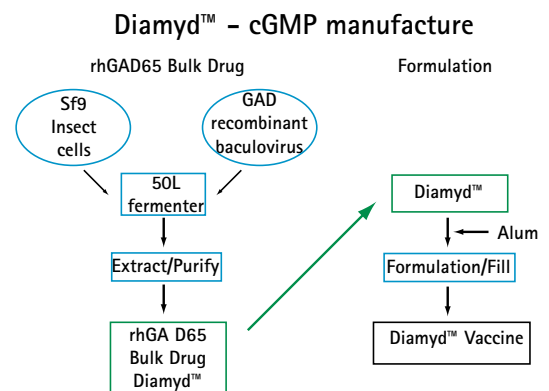
Our manufacturing process relies on the expression of recombinant human GAD65 in an insect cell line - grown under special conditions – after infection with an insect specific baculovirus containing the GAD cDNA (our “baculoGAD” recombinant clone). This is referred to as the baculovirus/insect cell expression system (or BVES). Both the quantity and quality of GAD manufactured by our BVES process have proven appropriate up to the current stage of development – and seem likely to meet our future requirements up to market introduction.

While currently at the 50 litre scale (with each 50L batch providing sufficient GAD for thousands of vaccinations) we have already found that our process can readily be scaled-up to 500 litres (providing tens of thousands of vaccinations per batch). So, pending the successful outcome of our Phase II, our manufacturing process seems capable of producing sufficient GAD for further clinical development and market introduction.

Apart from its suitability for manufacturing active GAD, the BVES also has inherent safety advantages – as a non-mammalian expression system modified to avoid contact with any mammalian components. This implies the low risk of contamination of our vaccine by harmful viruses. Moreover, because the vast majority of human viruses are not able to grow in the insect cells used, the likelihood of

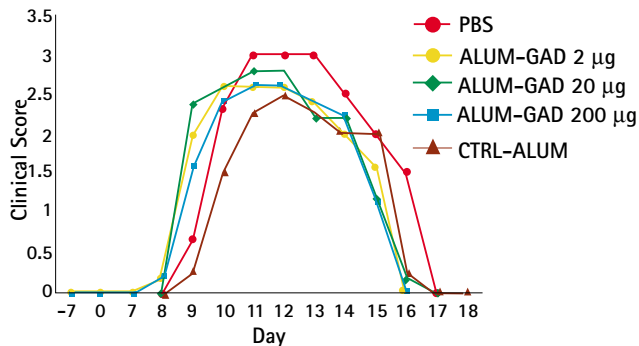
these inadvertently being propagated during manufacture and contaminating our vaccine is considerably reduced. Similarly, because the “baculoGAD” recombinant clone is an insect-specific virus (that can not infect mammalian cells) the risk imposed by possible residual traces of residual virus in our vaccine is greatly reduced.

A final attribute of our GAD therapeutic strategy has recently



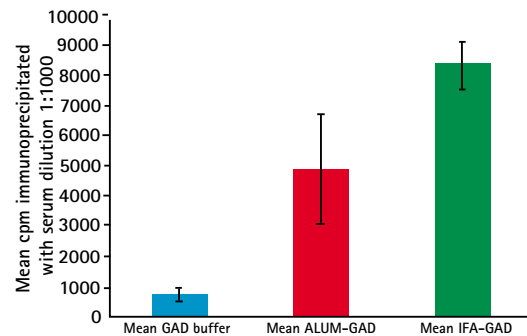
become apparent. Despite only one (or a few) small regions (ca. 12 amino acids) of GAD being thought as likely to be active, we made the decision at the outset (now 9 years ago) that we would not risk

No Impact on Experimental Autoimmune Encephalomyelitis Development



Assoc. Prof. Robert Harris CMM, Karolinska Institute, Sweden, 2000
 • Clinical Course of MBP 63-88 –EAE in Lewis rats

Immunogenicity of GAD is Increased with Use of Adjuvant



Prof. Åke Lenmark, University of Washington, Seattle, USA, 2000
 • Total rat serum IgG anti-GAD radioimmunoassay

choosing the “wrong” portion - and our vaccine would contain the full-length GAD protein (consisting of 585 amino acids). So, despite the obvious difficulties imposed in manufacturing a recombinant protein of this size (which would be avoided by synthesising a peptide instead) we decided that our vaccine would contain full-length, natural GAD protein - leaving antigen processing and presentation of the appropriate region (“determinant”) to that individual patients immune system. This decision not to pursue GAD peptide therapy seems to have paid off in view of recent scientific reports that repeat injections of protein fragments (“peptides”) can confuse the immune system and result in immune over-reaction (anaphylaxis) – that can be life threatening in experimental animals. This response is not shown by the respective (intact) proteins. Our use of the full-length GAD65 protein in our vaccine avoids this

Our initial manufacturing was conducted to the principles of GLP (“Good Laboratory Practice”) and was used successfully for all pre-clinical safety studies and clinical Phase I (in healthy volunteers). These ca. 25 different pre-clinical safety studies used various quantities of either GAD alone, or the GAD vaccine (alum-GAD), in several different species, and administered by different routes. All these studies followed international regulatory requirements to establish the pre-clinical safety after GAD administration, and thereby provided the basis for Phase II development. In contrast to the pre-clinical and

Phase I stages, however, the GAD vaccine used for Phase II is manufactured to the highest quality standard available for manufacture of clinical therapeutics. This is “cGMP” (or “current Good Manufacturing Practice”) – that will also be required for all future clinical development.

Diamyd’s development of the GAD vaccine has now culminated with completion of our Phase II clinical trial in 47 “Type 2” diabetes patients with GAD-antibodies (LADA). This study is truly pioneering – in that this is the first time patients have received the GAD vaccine.

Prior to un-blinding, I am highly optimistic regarding the outcome of this Phase II. I think this study will be a resounding success if the study outcome includes the following:

1. there are no safety concerns of alum-GAD vaccination
2. there is evidence for responses that are consistent with a positive therapeutic effect.



John Robertson, Ph.D., has been Director of Research Development in Diamyd Medical since 1994. Robertson has experience in biotechnology and toxicology, both from industry (Inveresk Research International, U.K. and Schering Agrochemicals, U.K.) and academic institutes (Karolinska Institute, Stockholm; The Pasteur Institute, Paris; and the NIH in Washington).

T cell GAD65

For use of GAD in immunological assays

bulk rhGAD65

For use of GAD in enzymatic assays



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