Targeting of calcitonin gene-related peptide expression to the pancreatic beta cells prevents insulin-dependent diabetes mellitus in non-obese diabetic mice

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TARGETING OF CALCITONIN GENE-RELATED PEPTIDE EXPRESSION TO THE PANCREATIC BETA CELLS PREVENTS INSULIN-DEPENDENT DIABETES MELLITUS IN NON-OBESE DIABETIC MICE

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A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

by

Armen Khachatryan

1996
Dedicated to my parents

how can a blade of grass return
the warmth received from the spring sun
I would like to thank and acknowledge my thesis advisor, Dr. Agnes Vignery, for her unending patience and unrelenting support, and the following persons for their contributions to this thesis project:

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1. ABSTRACT

To investigate whether the immunosuppressive neuropeptide calcitonin gene related peptide (CGRP) was a potential candidate for tissue-specific gene therapy, we engineered Non-Obese Diabetic (NOD) mice to produce CGRP in beta cells by placing the modified calcitonin gene under the control of the rat insulin promoter. CGRP inhibits the production of cytokines by Th1 cells which have been implicated in the pathogenesis of type I diabetes. Three transgene positive mouse lines were obtained, two of which express immunoreactive CGRP in beta cells (NOD-CGRP mice). Isolated islets from one of these two transgene positive founders produced active CGRP while islets from transgene negative mice did not. The production of CGRP by beta cells prevents IDDM in male, and reduces its incidence by 63 % in female NOD mice. This immunosuppressive effect of CGRP is due to a local effect and not a systemic effect of CGRP on peripheral lymphoid organs as no difference was detected between NOD-CGRP and NOD mice lymph node, spleen and thymus cells by either fluorescence-activated cell sorter analysis or proliferative response to stimulation by antigen, alloantigen or anti-CD3. CGRP partially prevented peri-insulitis in NOD-CGRP mice whose lymphoid cell composition was similar to that of NOD mice, further suggesting the local immunoregulatory effect of
CGRP. These data suggest that CGRP is a potential therapeutic molecule to prevent or treat diabetes and possibly other diseases and conditions in which immune cells are involved. These data also suggest that endogenous CGRP concentrated in sensory nerve endings may regulate locally in tissues the immune response, further strengthening the importance of the functional neuro-immune link.
2. INTRODUCTION

2.1 Establishment of Reference Point

\textbf{BALANCE + SIMPLICITY = EXISTENCE}

At a very basic level to defend a thesis, a reference point is required. As odd as it may seem, I have chosen the above equation as that reference point. Arguably the goal of life at a rudimentary level is existence. If this is the case, then I believe that the requirements of existence from an evolutionary point of view are balance and simplicity. During existence an organism is subjected primarily to two main forces: environmental and evolutionary, which are one and the same but on a different time scale. Environment mandates balance and evolution mandates simplicity.

Balance implies some arbitrary midpoint in between two extremes, between high or low, between much or little. Further implication is that an organism that is in balance can exist within the range of the two extremes and, if subjected to environmental forces, can shift its balance in one direction or the other to adapt accordingly. The time scale between the stimulus (an environmental force) and the response (shift in balance) is small.
Evolution, on the other hand, exerts its influence on a much larger time scale. Evolutionary change is determined by the functional efficiency (adaptedness) of the organism in its environment. Webster's dictionary defines efficiency as an "ability to produce a desired effect . . . with a minimum of effort, expense, or waste." Any given organism must perform numerous biological functions in order to survive. Each and every one of these functions must be performed with the least amount of effort, expense and waste. If ten competitors involved in a contest were asked to design a system to perform a certain function, surely the simplest design with the least number of steps would win, unless a different design, even if it involved a few more steps, could be shown to perform that function much better. Over billions of years every step of the evolution has been put to the same test by nature. A human being, which some may egocentrically believe to be the epitome of the evolutionary design, may seem quite complex at first glance. However, it is a large system comprised of indefinite number of simpler subsystems each performing its assigned function in the most efficient manner. It is of vital importance to realize that this is not a system whose mechanism can be explained by linear dynamics, rather it is a system comprised of extensive nonlinear networks which at every step simply self-regulate through a multitude of
feedback mechanisms. Finally, to paraphrase a well known saying, evolution is the survival of the fittest and the simplest.

Most if not all of science is the study of nature and of existence in particular. Through the ages nature has selected to travel down the path of simplicity and balance. Consequently, if we are to unravel and explain the “way of nature,” then we must abide by its rules and follow the same path it has taken. Following the gathering of data, teleologically we must strive to formulate the simplest possible theory consistent with all that is known and with balance as its fundamental tenet.

2.2 Autoimmune Disease

Understanding complex biological systems is a great challenge. Every discovery represents an individual piece of information that needs to be arranged in its respective place without knowing with certainty how the pieces interrelate. With this in mind theories are introduced as to what the overall picture may look like and how the individual pieces fit together. This is an essential step because just like in a jigsaw puzzle, even if all the pieces are present, if an incorrect picture is used as a guide, the puzzle cannot be solved. Various theories of how the immune system functions in a physiological setting have evolved within the past century.
We are taught that immunology is the science of self-nonself discrimination\(^2\). Yet the understanding of how the boundary between self and nonself is established is far from complete. What is certain is that under "normal" conditions self is tolerated while in an autoimmune disease, a breach in the self-nonself boundary takes place and self is no longer tolerated. To a great extent as a consequence of our incomplete understanding of immunophysiology, the definitive mechanism of autoimmune disease remains a mystery. However, enormous amount of work has been done in this field in the past half century.

Burnet, in 1959, proposed the "clonal selection theory" and according to this theory the immune system during its development consists of cells that possess within their repertoire receptors that are potentially self reactive. Self tolerance is established by a process of thymic deletion of self reactive T-cells, while cells that are reactive to foreign antigens are spared\(^3\). This process of self-reactive T-cell deletion has been confirmed as recently as 1987\(^4\). During healthy states only immune cells that are capable of potentially binding foreign antigens exist and recognition of self is nonexistent. Autoimmune disease is born when, for various reasons or purely by accident, elements of the immune system appear that recognize self and embark on a journey of self destruction\(^5\). In other words autoimmune disease must result whenever a cell clone that was
eliminated during thymic education enjoys a reincarnation of sorts. In contradiction to this theory, various authors have reported that both T and B cells capable of autoimmune disease induction have been detected in the peripheral lymphoid tissues of healthy individuals\textsuperscript{6,7}. Recently, Katz and his colleagues looked at the tolerance status of T cells bearing the transgenic, pathogenic T cell receptor (TCR) for self antigen responsible for insulin-dependent diabetes mellitus (IDDM) of the Non-Obese Diabetic (NOD) mouse model\textsuperscript{8}. T cells with the transgenic TCR were not deleted as would have been predicted by the clonal selection theory. In fact, they interacted with self-antigens both \textit{in vitro} and \textit{in vivo}, and induced IDDM in these mice.

Other observations, which also contradicted clonal selection theory, have led to the formation of alternative theories including the "idiotypic network theory." This theory is based on the hypothesis that the immune system consists of both idiotypic and anti-idiotypic entities which through their interactions establish immunologic homeostasis. Autoimmune disease results as a consequence of aberrant and disproportionate proliferation of certain clones. The roots of this theory can be traced back to Jerne who in 1974 proposed that self-reactivity is a normal component of the immune system whose major function is to maintain internal macromolecular homeostasis rather than protect the host from external invasion\textsuperscript{9}. 
Barnett in early 1980’s proposed the “primary lesion theory” which suggests that autoimmunity is not itself a distinct entity, but a physiological response to sustained antigen overproduction in diseased tissue, and fundamentally no different from the response to foreign antigen\(^\text{10}\). The phenomenon referred to as the “precipitating event in autoimmunity” is viewed as a primary lesion, whose continued presence is necessary to drive and sustain the response conventionally referred to as autoimmunity. Those who develop clinical disease are regarded as high responders to critical antigens generated in excess by the primary lesion. High responder status is determined by immune response, HLA-linked, genotype. The HLA class II (D region genes) are closely linked to immune response genes which control the response of specific clones\(^\text{11}\). Thus, one strain of animal may fail to respond to an antigenic shape to which another strain of the same species reacts strongly. HLA phenotype is a good marker for such differences, and what applies to exogenous antigens also applies to autoantigens, but over a range of reactivity normally consistent with health. Barnett suggests that in type 1 diabetes, a primary rise in \(\beta\)-cell antigen load is met with an expansion of specific and appropriate clones. The extent of clonal expansion relative to the antigen overload is HLA determined. Effector immune cells cannot distinguish diseased from healthy \(\beta\)-cells, and in the minority of those with the primary lesion who are high responders,
their greater number leads to inexorable destruction of all β-cell tissue.

Since cell products in excess are toxic and biologically active substances can cause various clinical syndromes, Barnett hypothesizes that "physiologic" autoimmunity may act as a defense mechanism in the face of antigen excess not only by neutralizing their biological or toxic effect on contact but by providing an alternative pathway for their clearance. Thus, although autoimmune disease may sometimes lead to organ destruction and death of the individual, it may have an advantageous defensive function for the species.

All three of the theories discussed above attempt to explain the process of tolerance. A large body of evidence exists that both supports and contradicts these theories. Though the simplest, the clonal selection theory, because of its suggestion that autoreactive cells are categorically deleted during thymic education, is certainly lacking in balance. The idiotypic network theory, though more complex, incorporates balance as its fundamental building block. In fact, it goes hand in hand with the primary lesion theory. As idiotypic network theory suggests the immune system is utilized by the organism not only to ward off foreign invasion, but also to maintain internal homeostasis. When certain clones within the network disproportionately proliferate, they do so as a result of a
certain precipitating event. This precipitating event is viewed as the cornerstone of the primary lesion theory. Taken together, the idiotypic network theory and the primary lesion theory suggest a mechanism of tolerance and breakdown thereof, that lies within the realm of both simplicity and balance.

2.3 Diabetes Mellitus

2.3.1 Overview of Diabetes

Diabetes is the name given to a constellation of symptoms arising from hyperglycemia. The disease can occur as a result of specific secondary causes ranging from iron overload of beta cells seen in hemochromatosis to insulin receptor defects. However, vast majority of patients with diabetes do not have a specific identifiable defect and are said to have idiopathic diabetes.

It was little more than 20 years ago that diabetes was thought of as a single disease with more or less severe manifestations. The clinical distinction of more severe, ketosis-prone, insulin-dependent type (IDDM) and the more common, non-insulin-dependent type (NIDDM) had been recognized by clinicians for many years. In 1951, Lawrence and his colleagues proposed that diabetes could be divided into two main types: "[1] Those who are probably not insulin-deficient and [2] those who certainly are." This observation was based on the studies that they performed on 10 diabetics: five young ketotic patients who they believed had insulin deficiency and five obese non-
ketotic patients in whom diabetes was attributed to factors other than insulin deficiency\textsuperscript{12}. Insulin level in these patients was estimated by injecting the patient’s plasma into alloxan-diabetic hypophysectomized and adrenalectomized rats. The fall in the blood glucose of the rats after 1 hour correlated with the amount of insulin. The five patients with ketosis had no insulin unlike the five non-ketotic patients who did.

The first suggestion of significant difference between the two types diabetes came from identical twin studies. It was found that concordance was nearly one hundred percent in non-insulin dependent diabetics, but less than fifty percent in insulin dependent diabetics\textsuperscript{13}. The proof of fundamental difference between the two types of diabetes came from a study published in 1974 that showed a strong association between certain HLA types and IDDM, but no such association was observed in NIDDM\textsuperscript{14}. The authors believed that IDDM had an autoimmune basis and that viruses were also involved in the disease pathogenesis as was suggested by Gamble and his colleagues in earlier studies\textsuperscript{15}. In that same year Bottazzo reported antibodies toward normal pancreatic islets cells in 13 patients with multiendocrine deficiencies\textsuperscript{16}. All these observations were in concordance with reports made in 1950’s and 60’s regarding the presence of the inflammatory infiltrates in the pancreas of insulin-dependent diabetics\textsuperscript{17,18}. In short, all these studies and many more
that have followed in the recent past have played a key role in the establishment of the current thinking that IDDM is caused by complete, autoimmune destruction of the beta cells of the pancreas.

A summary of the main clinical characteristics of the two types of diabetes is presented in Table 1. In IDDM, hyperglycemia and ketosis appear suddenly in non-obese children or young adults and require chronic therapeutic intervention with small doses of insulin. The typical pathologic findings include insulitis followed by islet cell destruction. A strong association exists between IDDM and HLA DR3 and DR4. On the other hand, NIDDM patients tend to present at an older age with symptoms of more chronic nature, are often obese and can be treated with dietary restrictions. In certain fraction of non-insulin-dependent diabetics amyloid deposits are found. Genetic predisposition is believed to be strong, however, no association with HLA types has been observed. In all populations NIDDM is much more common than IDDM, with the risk of developing NIDDM being more than 30 times greater\textsuperscript{19}.

2.3.2 Type 1 or Insulin-Dependent Diabetes Mellitus

Following the establishment of IDDM as a fundamentally different disease from NIDDM, substantial body of research has been published on its etiology and pathogenesis. What is certain about the etiology of IDDM is that it involves a complex interplay between a
number of genetic and environmental factors. Genetic factors play an essential role as evidenced by the involvement of both MHC and non-MHC predisposition factors. In Caucasians increased prevalence of IDDM is seen in individuals with HLA DR3 and DR4 haplotypes. Furthermore, individuals who are DR3/DR4 heterozygotes have a twenty to forty fold higher risk than the general population\textsuperscript{20}. In contrast, reduced prevalence is found in individuals with HLA DR2 haplotype\textsuperscript{21}. Other MHC associated genes associated with IDDM protection include Tap-1 and Tap-2 genes which code for transporters of antigenic peptides to MHC class I molecules\textsuperscript{22}.

**Table 1: Main clinical characteristics of the two types of diabetes (from “Insulin” Aschroft and Aschroft. (1992) IRL Press.)**

<table>
<thead>
<tr>
<th>Type of Diabetes</th>
<th>Insulin-Dependent-Diabetes Mellitus</th>
<th>Non-Insulin-Dependent Diabetes Mellitus</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age of Onset</strong></td>
<td>Juvenile Onsetage</td>
<td>Maturity Onset</td>
</tr>
<tr>
<td>age, 0-30 years</td>
<td>usually 35 years or more</td>
<td>usually obese</td>
</tr>
<tr>
<td>non-obese</td>
<td>usually obese</td>
<td>often symptoms for years</td>
</tr>
<tr>
<td>short history</td>
<td>often symptoms for years</td>
<td>moderate hyperglycemia</td>
</tr>
<tr>
<td>severe hyperglycemia</td>
<td>rarely ketotic unless major</td>
<td></td>
</tr>
<tr>
<td>often ketotic</td>
<td>infection</td>
<td></td>
</tr>
<tr>
<td><strong>Therapeutic Requirements</strong></td>
<td>requires insulin therapy</td>
<td>can be treated with diet or tablets</td>
</tr>
<tr>
<td>insulin sensitive (small doses of insulin needed)</td>
<td>insulin resistant (large doses of insulin needed)</td>
<td></td>
</tr>
<tr>
<td><strong>Pathology</strong></td>
<td>Type I diabetes</td>
<td>Type II diabetes</td>
</tr>
<tr>
<td>islet cell antibodies present</td>
<td>islet cell antibodies absent</td>
<td></td>
</tr>
<tr>
<td>beta cells destroyed by immune inflammatory cells</td>
<td>often amyloid in islets</td>
<td></td>
</tr>
<tr>
<td><strong>Genetic Predisposition</strong></td>
<td>low concordance among identical twins associated with HLA DR3 and DR4</td>
<td>high concordance among identical twins</td>
</tr>
</tbody>
</table>
Apart from the insulin gene, the search for the candidate non-MHC predisposing genes has been rather unfruitful in human IDDM. The 5' region of the insulin gene has been shown to be associated with IDDM, independent of parental transmission and HLA type. Heterozygosity of the beta chain of the T cell receptor (TCR) was reported in 1987 to have an association with IDDM, however since then there has been no convincing evidence that TCR genes influence susceptibility to disease. Studies of the NOD mouse have led to the description of 10 non-MHC predisposition loci, in addition to the major association with MHC loci. These are discussed in more detail elsewhere in this paper under the subheading of NOD mice.

Many of the studies described above establish genetic susceptibility as a required but not sufficient component of the disease process. As monozygotic twin studies have shown only 30-50% concordance of IDDM, it is environmental influences that provide the link between the diathesis and the disease. The importance of environmental factors are well established in animal models of spontaneous diabetes. In NOD mice variation of the diet or several viral infections reduce disease susceptibility.

In human populations it is much more difficult to establish specific direct links between environmental influences and the development of IDDM. However, ample evidence exists that supports this hypothesis. Disease frequency varies greatly from country to
country with the greatest incidence found in Scandinavian countries. It seems that in certain colder climates IDDM is found at a greater frequency. This is quite interesting, since it has been shown that by raising the ambient temperature the incidence of diabetes can be reduced in the NOD mice. Studies of migrants from countries with low IDDM frequency to countries with high frequency have shown that they become more susceptible to disease in their new environment. Other data from stable populations in Europe has shown a sudden surge in incidence of IDDM. This change in Europe represents a two to three-fold increase over a period of 30 years and is clearly evidence that cannot be explained on genetic basis alone. Some of the environmental factors implicated as possible triggers of IDDM include viruses, most notably Coxsackie B virus and cytomegalovirus, bovine albumin found in cow’s milk, and even stress. The suggested hypothesis is that both Coxsackie B viral protein and bovine albumin share a sequence with the beta cell autoantigens, glutamic acid decarboxylase (GAD) and p69, respectively, and by virtue of molecular mimicry, immune reactions directed against the xenoantigens cross react with the beta cell autoantigens.

The etiologic role of the environmental and genetic influences seem certain, but the nature and the extent of the contributions that each makes to the development of disease still requires much work.
Once the events that lead up to the break down of self tolerance have taken place, the details of the effector autoimmune cascade that follow seem clearer. Even though anti-islet cell antibodies have been demonstrated both in rodent and human IDDM, there is no evidence that these autoantibodies are pathogenic since the disease cannot be transferred by the serum of the affected mice. Without a doubt IDDM is a T-cell dependent autoimmune disease characterized by infiltration and destruction of the pancreatic islets by the cells of the immune system. Diabetes can be transferred to normal syngeneic animals by purified T cells from diabetic NOD mice. On the other hand diabetes can be prevented in NOD mice by anti-TCR monoclonal antibodies which selectively eliminate T cells. The two subsets of T cells, CD4+ and CD8+, play different roles in this process (Figure 3). CD4+ T cells play an important role in both the recruitment and the activation of the immune effector cells. Lymphokines produced by CD4+ T cells include Interleukin-2 (IL-2), Interleukin-3 (IL-3), Interferon-γ (IFN-γ), Tumor Necrosis Factor-α (TNF-α), Tumor Necrosis Factor-β (TNF-β) and Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF). IFN-γ, TNF-α and TNF-β have been shown to act as mediators of adhesion and extravasation for various inflammatory cells. IL-2 induced proliferation of CD8+ T cells is one of the most crucial events in the pathogenesis of IDDM, for it is the army of CD8+ T cells that delivers
the major "cytotoxic" blow to the β-cell of the pancreas\textsuperscript{43}. In addition to T cells, macrophages as well have been implicated in the destruction of the beta cells via local cytokine or free radical release. IL-1, which is released by activated macrophages, has been shown to have a cytotoxic effect on the pancreatic islets\textsuperscript{44}.

IDDM is clearly an autoimmune disease as reflected by the evidence given above, which represents a minute fraction of the existing body of scientific knowledge. Clear understanding of the etiology and the pathogenesis of IDDM still eludes us. However, given the astounding pace of modern science, the proximity of this presently elusive goal seems certain.

2.4 Calcitonin Gene Related Peptide (CGRP)

CGRP was first described by Rosenfeld and his colleagues in 1983\textsuperscript{45}. The story behind the discovery of this novel neuropeptide is quite interesting. Most peptides come to our attention as a result of certain biological function that they perform. The usual sequence of events include the purification of the peptide, followed by amino acid sequencing and lastly the identification of the gene that codes for the peptide. In the case of CGRP, everything was reversed as a consequence of recombinant DNA technology (Figure 4).
While Rosenfeld and his colleagues were studying the calcitonin gene transcription, they unexpectedly found a second, structurally distinct transcript, which they referred to as calcitonin gene related peptide mRNA. Through isolation and sequence analysis of the calcitonin genomic DNA, and calcitonin and CGRP cDNAs, they discovered that both CGRP and calcitonin mRNAs were generated by differential RNA processing from a single genetic locus (Figure 5). In the thyroid C cells the primary RNA transcripts are processed to mRNA for calcitonin, while mRNA for CGRP is formed predominantly in neural tissue. Since its discovery, CGRP has become a subject of extensive research which has revealed the peptide's important implications as a chemical messenger of the nervous system.
CGRP is co-localized and co-released with substance P in sensory nerves\(^4^8\) and with acetylcholine in motorneurons\(^4^9\). In the brain, CGRP is broadly distributed and especially concentrated in hypothalamic areas and some brainstem nuclei\(^5^0\). CGRP is ubiquitous in the peripheral tissues, including the digestive system\(^5^1\). It has been detected in the blood stream\(^5^2,5^3,5^4\) and in the heart, predominantly in the atria, around coronary vessels and in the myocardium\(^5^5\).

A variety of biological effects have been attributed to CGRP, including the modulation of nicotinic receptor activities at the neuromuscular junction\(^5^6\) and of substance P in inflammation\(^5^7\), a reduction of gastric acid secretion\(^5^8\), potent peripheral blood vessel
dilation\textsuperscript{59,60,61}, increased proliferation of human endothelial cells\textsuperscript{62}, cardiac acceleration\textsuperscript{63}, a regulation of calcium metabolism\textsuperscript{64} and insulin secretion\textsuperscript{65}, an increase in body temperature, and a decrease in food intake\textsuperscript{66}. Dr. Vignery, and her colleagues, here at Yale, have demonstrated that CGRP has potent immunosuppressive activity in \textit{vitro} by virtue of inhibiting the production of IL-2 by T cells\textsuperscript{67}.

The activation of T lymphocytes leads to the production of IL-2, a lymphokine which initiates a cascade of cellular events. Among the cytokines produced by immune cells, IL-2 is the earliest growth factor produced by T cells upon stimulation by antigen and iL-1. IL-2 is not only a potent T and B cell mitogen but also a direct activator of natural killer cells. Thus, the production of IL-2 is a critical step that influences the immune response.

By virtue of its capacity to induce the proliferation and the differentiation of multiple T cell subsets as well as the release of cytokines, IL-2 plays a key role in the initiation and evolution of the immune reaction that leads to tissue remodeling or repair. In as much as the production of IL-2 has beneficial effects in immune defense mechanisms, its production can also be associated with tissue destruction that accompanies chronic inflammatory reactions and autoimmune diseases. Thus, local control mechanisms may help regulate immune reactions.
Dr. Vignery has demonstrated that T lymphocytes express functional receptors for CGRP which prevents the production of IL-2 by a cAMP-mediated inhibition of IL-2 gene expression\(^6\^7.\)

From the evidence that has been presented in this section, it appears that CGRP plays both a regulatory and modulatory role along the neurovascular, neuroendocrine and neuroimmune axis.

### 2.5 Non-Obese Diabetic (NOD) Mouse Model

The NOD mouse represents a model in which autoimmunity against beta cells is the primary event in the development of insulin-dependent diabetes mellitus (IDDM). It serves as one of the best characterized and most widely used models of autoimmune diabetes. The NOD mouse was derived from a cataract developing substrain of outbred ICR/JC1 mice by selective breeding at Shianogi Laboratories in Japan in the early 80's. Since then many substrains of NOD mice have been established which differ in diabetes incidence and time of onset. Typically 80-100% of female mice and 15-40% of male mice develop the disease between 3 to 6 months of age. In these mice the sex difference is attributed to the sex hormones, since castration increases the incidence of diabetes in males and decreases the incidence in females. Environmental factors, such as diet\(^2\^5,\) can influence the rate of development of the disease. Furthermore, animals protected from infections by maintenance in a pathogen free
cages have higher incidence of diabetes. Crowding of mice and frequent handling also elevates the incidence of diabetes, and this is believed to be due to stress.

IDDM is a disease whose main clinical feature is hyperglycemia. In NOD mice this is easily determined by whole blood portable glucose monitor. However, in initial screenings it is more practical and less expensive to measure glucose levels in urine, since hyperglycemia inevitably leads to glycosuria. When persistent glycosuria is observed, then more accurate portable glucose monitoring can be initiated.

The pathologic lesion leading to IDDM in both NOD mice and in humans is the autoimmune destruction of the insulin-producing beta cells in the pancreas. The course of the disease in mice can be followed by histological examination of the surgically removed pancreas. A typical longitudinal histologic examination demonstrates the following: at 3-4 weeks of age, infiltrating immune cells surround the blood vessels (perivascular infiltration) but the islets are still clear. At 6-7 weeks, the infiltrating cells reach the islets (peri-islet infiltration). Between 10-12 weeks, the infiltrating cells penetrate into the islets (intra-islet infiltration) and the islets become swollen with lymphocytes. Following the onset of overt diabetes at 14-20 weeks, the infiltrate disappears, leaving the islets greatly reduced in size and devoid of beta cells.
By staining with antibodies directed at markers of T cells, B cells, and macrophages, it has been shown that macrophages predominate among the cells first infiltrating, followed by CD4 T cells, then by CD8 T cells and B cells. Experiments employing adoptive transfer of T cells have shown that CD4 T cells from prediabetic NOD mice did produce insulitis following the transfer, but did not lead to islet destruction or hyperglycemia; the transfer of CD8 T cells did not produce insulitis or hyperglycemia. However, when CD8 T cells were transferred together with, or after CD4 T cells, autoimmune diabetes developed. Taken together with the data from the immunohistochemical analysis of the lesions, it seems that CD4 T cells home to the islets, attach themselves and proliferate, and enable the later-arriving CD8 T cells to reach the islets and engage in the destruction of the beta cells. Antibodies do not appear to play any role in the pathogenesis of IDDM in either humans or mice, even though the presence of several antibodies have been detected before the onset of overt diabetes.

The development of diabetes in NOD mice is under the control of multiple genes. At least ten genes, both MHC and non-MHC related, mapped to nine different chromosomes have been shown to contribute to the susceptibility of NOD mice to diabetes. Susceptibility gene Idd-1 is located in the murine MHC on chromosome 17, and is a gene complex with two susceptibility loci.
the class II genes I-A\(\beta\) and I-E\(\alpha\). I-A\(\beta\) gene is homologous to the human DLADQ1B1*0302 IDDM-associated allele in having serine instead of aspartate at position 57\textsuperscript{4}. NOD mice do not express the other MHC class II product, I-E, owing to the mutation in the I-E\(\alpha\) gene. The absence of the I-E product is necessary for NOD diabetes. The introduction of a normal I-E\(\alpha\), as a transgene in NOD mice, completely prevents diabetes\textsuperscript{75}. However, evidence contrary to this was later presented by Podolin and Wicker indicating that the expression of I-E in NOD mice is not in itself sufficient to prevent diabetes\textsuperscript{76}.

The non-MHC susceptibility genes that play a role in NOD diabetes have been designated Idd-2, Idd-3, Idd-4, Idd-5, Idd-6, Idd-7, Idd-8, Idd-9 and Idd-10, and mapped to Chromosomes 9, 3, 11, 1, 6, 7, 14, 4, and 3 respectively. The nature of each of the non-MHC susceptibility genes is less well defined in their effect on the disease.

Development of diabetes is mediated through a multifactorial interaction between MHC class II genes and multiple, unlinked, genetic loci. Moreover, the NOD mouse demonstrates the critical interaction between heredity and environment. The difference in disease incidence and age of onset among various colonies, depending on the cleanliness of the housing conditions, illustrates how environmental factors can affect the penetrance of diabetes-susceptible genes.
Lastly, one should bear in mind that IDDM patients are genetically and probably pathogenically heterogeneous. Thus, NOD mice might represent only a part of the IDDM population. Use of an animal model to study human disease always requires cautious interpretation.

2.6 Rat Insulin Promoter and Transgenic Technology

Transgenic technology allows the scientist to incorporate and express a given gene in a specific tissue or cell type. In part, this is possible because of the remarkable ability of selective expression of specific genes by differentiated eukaryotic cells. For example, the beta cell of the pancreas is the only cell in the body that can express insulin even though the genetic information for expression of insulin is present in all the cells of the organism. The information of whether insulin or any other protein will be produced in the pancreas or in the brain is not contained within the coding sequence of the insulin gene: rather it is contained in a number of distinct transcriptional control elements located upstream of the mRNA initiation site. These transcriptional control elements collectively are referred to as the promoter region. Through recombinant DNA techniques a given gene could be attached to another promoter
region which would result in the expression of the given gene only in the cells where the promoter has a permit of a sort to act in.

In the transgenic mouse that we created, the CGRP gene was linked to the rat insulin promoter, which would insure the targeted expression of CGRP to the beta cells of the pancreas. Along with the development of the transgenic technology in mid 80's, the rat insulin promoter, which was first described in 1979 by Lomedico and colleagues\(^7\), has been used to express several cytokines in the beta cells.
3 MATERIALS AND METHODS

3.1 Reagents

Human CGRP, CGRP8-37, mouse amylin and human CGRP RIA were purchased from Peninsula Laboratories (Belmont). Complete Freund adjuvant and M-MLV reverse transcriptase were purchased from Gibco-BRL (Grand Island). Keyhole Lympet Hemocyanin (KLH) was purchased from Calbiochem (La Jolla). Unless otherwise stated, all reagents were obtained from Sigma (St Louis)

3.2 Antibodies

The following Abs were used: guinea pig anti-human insulin (BioGenex, San Ramon), mouse anti-porcine glucagon (Sigma, St Louis), rabbit anti-human CGRP (Penninsula, Belmont), biotinylated rat mAb anti-mouse Ly5 (B220) (Caltag, South San Francisco, CA), biotinylated rat mAbs anti-mouse CD4, CD8a and CD4+red613 (1/500) (GibcoBRL, Grand Island), biotinylated anti-mouse CD8+ (TIB105, ATCC), anti-mouse CD4+red613 (1/500) (GibcoBRL, Grand Island, NY), anti-mouse B220+ and avidin-phycoerythrin (PharMingen, San Diego), anti-mouse-TCR+FITC (H57-557, ATCC), donkey anti-guinea pig-FITC and -Cy3Tm, donkey anti-mouse-FITC and -Cy3Tm, goat anti-rabbit-Cy3Tm (Jackson ImmunoResearch Lab, Inc., West Grove), streptavidin-phycoerythrin (Calbiochem, La Jolla, CA), goat anti-mouse Ig (H+L) coupled to magnetic beads (Biomags,
8-4340G, Cambridge), hamster mAb anti-mouse CD3e (2C11-145, ATCC).

3.3 Cells

Cytotoxic T-Cell Line (CTLL) and T cell lymphoma EL-4 were obtained from ATCC (Bethesda).

3.4 Construction Of The pRIP-hCGRP-Ex2 Transgene

The human CGRP gene cloned into pGEM4 plasmid vector (Promega) was kindly provided by Dr. Gilbert Cote from MD Anderson Cancer Center in Houston, Texas. This construct was named pGEM4-CGRP. The TGCA splice site upstream of Exon 4 was deleted. The resulting gene preferentially leads to the production of CGRP mRNA as shown in Figure 5. pBS-SK-RIP plasmid vector containing the rat insulin promoter was kindly provided by Dr. Dominique Picarella from Howard Hughes Institute at Yale University School of Medicine. CGRP gene was excised out of pGEM4-CGRP using Hind III restriction enzyme, agarose gel purified, and ligated into the Hind III site of pBS-SK-RIP (Figure 6). The ligated DNA was transformed into DH5-a competent cells by electroporation using Bio Rad Electroporator (Settings: Capacitance 25 microFad, Resistance 200 ohms, Voltage 2.5 V). Electroporated cells were grown in 1ml SOC (LB broth rich in glucose) medium for 1 hour at 37° C, and 150ul was plated onto
Ampicillin/agar plates and incubated overnight at 37°C. 48 colonies were picked and cultured overnight at 37°C in LB+Ampicillin medium. DNA plasmid miniprep was performed on all 48 cultures using the "boiling method" described by Maniatis. 10 ul from each DNA plasmid miniprep was digested with Hind III to determine which sample contained the CGRP gene insert. To ensure the correct orientation of the inserted CGRP gene, plasmids containing the CGRP insert were subjected to further digests by Nhe I, Xmn I, Afl II, Apa I, Kpn I, and Spe I. We had therefore constructed the pBS-SK-RIP-hCGRP (Figure 6). As discussed in the background section on CGRP the exon 1 of the Calcitonin gene is non-coding and only serves as a regulatory element which could interfere with the insulin promoter driven targeted expression of CGRP in the beta cells of the pancreas. Thus, the sequences upstream of BstB I site, which included Exon 1 and Intron 1, were removed from the construct as follows. pBS-SK-RIP-hCGRP was double digested with BstBI and EcoR I. The digest was run on a 1% agarose gel, and the larger fragment, which contained the pBS-SK, RIP and Human CGRP from exon 2 onward, was purified by electroelution as described in Maniatis. The ends of the purified fragment were ligated using a 27 base-polylinker (EcoRI-EcoRV-XbaI-BstBI, obtained from oligonucleotide synthesis services at Boyer Molecular Biology Center). Following ligation, DNA was
transformed into DH-5a competent cells by electroporation. grown in overnight
Figure 6: A: pBluescript SK (pBS-SK) plasmid. B: pBS-SK-RIP constructed by inserting rat insulin promoter (RIP) into pBS-SK between BamHI and EcoRI sites. C: pBS-SK-RIP-hCGRP constructed by inserting CGRP gene into pBS-SK-RIP at Hind III site. D: pRIP-hCGRP-Ex2 constructed by excising Exon 1 and Intron 1 with EcoRI and BstBI restriction endonucleases and ligating the ends of the larger fragment with EcoRI - EcoRV - XbaI - BstBI polylinker.
cultures, and minipreped as described above. This construct was named pRIP-hCGRP-Ex2 (Fig. 6). To insure the integrity of the new construct, it was subjected to partial restriction map analysis using EcoR I, Hind III, Xba I, BstB I, Xmn I and BssH I.

pRIP-hCGRP-Ex2 was "maxipreped" and precipitated in CsCl column twice as described in Maniatis\textsuperscript{78}. Ethidium bromide from purified DNA was extracted using water saturated Butanol. Plasmid DNA was precipitated with EtOH and resuspended in TE buffer. 100ul of the plasmid DNA was digested with Not I and Hind III to isolate the RIP-hCGRP-Ex2 sequence from the pBS-SK vector. The separation was performed on 1% agarose gel and the RIP-hCGRP-Ex2 fragment isolated by dialysis. The isolated fragment was further purified using Elutip-d DNA column (CalBiochem). To desalt the DNA samples prior to injection into mouse embryos, they were dialysed with Millipore 0.05 micron round filter paper. DNA was quantified and diluted to the concentration of 5 ng/ml in sterile double distilled water.

2 picol of this 5ng/ul RIP-hCGRP-Ex2 were injected into fertilized eggs from NOD mice, and implanted in the uterine horns of pseudopregnant BALB/c female mice using standard procedures\textsuperscript{79}. 
3.5 Screening of RIP-hCGRP-Ex2 Transgenic Mice and Establishment of Colonies

3.5.1 Genomic DNA Preparation:

Solutions: 
1. Tail buffer - 50 mM Tris pH 8.0, 100 mM EDTA, 100mM NaCl, 1% SDS
2. Saturated phenol/chlorophorm/isoamyl alcohol (ratio 25:24:1)
3. Chlorophorm/isoamyl alcohol (ratio 24:1)

Approximately a 5 mm portion of the tails from 2 week old mice were excised. 700 ul of tail buffer and 25 ul of Proteinase K (10mg/ml) were added to each of the tails in an 1.5 ml eppendorf tube and incubated overnight at 55°C. DNA was isolated by two phenol/chlorophorm extractions, followed by a single chlorophorm extraction. 600 ul of ice cold EtOH was added to the aqueous phase which was then precipitated in a microcentrifuge at 14,000 rpm for 30 minutes. The supernatant was discarded, the pellet washed with 70% EtOH and suspended in 100 ul of H₂O. DNA concentration was determined using a spectrophotometer. A reading of 1.0 optical density (O.D.) at 260 nanometer wavelength represents to 50 ug/ml of double stranded DNA.
3.5.2 Tail DNA Analysis by Southern Hybridization:

Solutions: Recipes for all of the following solutions described in Maniatis\textsuperscript{78}.

1. 20 x SSC

2. Denhardt’s Solution

3. Hybridization buffer - 6 x SSC, 0.5% SDS, 0.01M EDTA, 5x Denhardt’s Solution, 100 µg/ml denatured Salmon Sperm DNA.

4. Denaturing Solution

5. Neutralizing Solution

10 µg of each tail DNA sample was digested with Pvu II and run on a 1% agarose gel. The gel was then bathed in 0.25 M Hydrochloric acid solution for 20 minutes. It was then placed in denaturing solution for two 20 minute intervals, followed by two 20 minute intervals in neutralizing solution. DNA was then transferred to a nitrocellulose membrane. Following the transfer, DNA was crosslinked to the membrane by ultraviolet crosslinker. This filter was prehybridized in hybridization buffer for 2 hours at 65°C. A RIP-CGRP probe, generated by excising a 647 bp fragment from the RIP-CGRP-EX2 plasmid using Xbal, was used to identify transgene positive mice. This probe was denatured by boiling for 5 minutes at 95°C, was then added to the hybridization buffer and incubated overnight at 65°C in a shaking water-bath. The filter was washed
successively with 3 x SSC + 0.1% SDS at 65°C two times, followed by 0.3 x SSC + 0.1% SDS twice, and eventually 0.1 x SSC + 0.1% SDS at 65°C. After the final wash was performed the filter was wrapped in SaranWrap and exposed onto a film with an intensifying screen at -70 C for 36 to 48 hours.

3.6 Immunohistology

Tissue immunostaining was undertaken for two reasons: (1) to ensure that CGRP was being produced in the beta cells of the pancreas and (2) to check whether CGRP was aberrantly expressed in any other peripheral organ. Pancreata were collected from F₀ NOD-CGRP and NOD mice, fixed at 4 °C overnight in periodate / lysine / paraformaldehyde fixative (Ref PNAS 18), processed sequentially through graded sucrose solutions [10%, 20%, and 30% (wt / vol)], and snap-frozen in tissue-Tek OCT (One-Touch, Miles (West Haven) by submersion in 2-methylbutane (EM Science, Gibbstown). 8-10 mm thick serial sections were collected onto gelatin-coated slides (Superfrost/Plus, Fisher Scientific, Pittsburg) and blocked with a solution containing 0.6 % Triton X-100 / 33% normal goat serum / 0.9 M NaCl / 0.2 M phosphate buffer, pH 7.4 for at least 2 hr at rt. The sections were then washed 3 times with a solution containing 0.3% Triton X-100 / 4 M NaCl / 0.2 M phosphate buffer prior to being incubated with
rabbit anti-human CGRP (1/2000) for 2.5 hr at rt. This polyclonal antiserum was pre-incubated with 20 mg / ml of mouse amylin to eliminate its cross-reactivity with amylin. The sections were washed 3 times as described above and incubated for 1.5 hr at rt with goat anti-rabbit-CY3™ (1:100).

For the histoimmunochemical analysis of pancreatic islets from the NOD and NOD-CGRP colonies, half pancreata were fixed for 24 hr in 10% phosphate-buffered formalin, paraffin-embedded, serially sectioned and stained with hematoxylin and eosin. The presence of mononuclear infiltrates, in and around capillaries and islets of Langerhans, along with the percentage of islets with peri-insulitis were recorded on twenty sections selected every 5 sections. The other half pancreata were processed for immunocytochemistry as described above. The primary antibodies used were as follows: guinea pig anti-insulin (1/50), mouse anti-glucagon (1/200), rabbit anti-human CGRP (1/2000), biotinylated rat mAbs anti-mouse Ly5 (B220) (1/20), CD4 (1/20), or CD8a (1/20). The sections were washed 3 times as described above and incubated for 1.5 hr at rt with the appropriate secondary antibody as follows: donkey anti-guinea pig-FITC or -Cy3™ (1/100 and 1/200 respectively), donkey anti-mouse-FITC or -Cy3™ (1/100 and 1/200 respectively), goat anti-rabbit-Cy3™ (1/100), or streptavidin-phycoerythrin (1/200). The surface area of the lymphocyte infiltrate around each islet was
estimated morphologically using the non-specific labeling of the nuclei by fluorescein. The percentages of CD4, CD8 and Ly5 (B220) lymphocytes, labeled with phycoerythrin conjugate, was estimated and expressed as percent of total immune infiltrate.

3.7 Islet Isolation and Culturing

Solutions: 1. Hanks Balanced Salt Solution (HBSS) - For every 100 ml of HBSS use 1 ml penicillin-streptomycin (Gibco), 3.25 ml Hepes (Gibco), 2 ml of 7.5% Bovine Serum Albumin (Gibco), 1 mg DNAse (Boehringer Mannheim)

Islets were isolated from the pancreata of freshly sacrificed mice. To digest the pancreatic tissue, it was treated with 5 ml of HBSS for 5 minutes on ice. Supernatant was removed and 5 ml of 0.075% of collagenase in HBSS was added and shaken for 5 additional minutes. Above steps were repeated for two more times. After the third shake 5 ml of Bruff's + 5% Fetal Calf Serum was added to each vial and incubated for 5 minutes at room temperature. This was a washing step and was repeated two more times. Following the final wash the vials were left in the washing solution and placed on ice until the the next step when islets were picked. Under the microscope the islets were identified as suspended white spheres, picked with a narrow tipped glass pipets, and placed in a clean petri
dish. The islets were cultured in Bruff's + 5% Fetal Calf Serum media.

3.8 Bioactivity and Proliferation Assay for CGRP

The murine T cell line EL-4 produces IL-2 in response to activation with PMA (ATCC). This line does not produce CGRP and CGRP inhibits its production of IL-2 in response to Phorbol 12-Myristate 13-Acetate (PMA). Figure 7 graphically illustrates the principle behind the bioassay. EL-4 T cells are cultured for 24 hours in the presence of PMA.
Figure 7: EL4/IL2 Bioassay Principle. A: Phorbol 12-Myristate 13-Acetate (PMA) stimulates EL-4 T cells to produce IL-2 which in turn stimulates CTLL cells to proliferate. B: CGRP decreases IL-2 production by EL-4 T cells. Decreased IL-2 production translates into decreased CTLL proliferation.

(5ng/ml) and log2 dilutions of supernatant to be assayed for CGRP activity. Positive controls include medium supplemented with increasing concentrations of rat CGRP. Supernatants from EL-4 T cells are added to CTLL, a mouse cytotoxic T cell line whose proliferation depends on IL-2. Extent of proliferation is measured by the amount of [³H] Thymidine incorporation by CTLL. A dose-dependent response analysis of CTLL cells to recombinant human IL-2 is performed in each assay to define the IL-2 standard curve and to determine the concentration of IL-2 produced by EL-4 T cells and therefore estimate the potency of CGRP.
3.9 Breeding of Mice

Mice were bred and kept in sterile filtered cages. For breeding purposes a positive transgenic male mouse was always placed in the same cage with two negative transgenic females and if successful impregnation was not observed within three weeks, different transgenic negative females were placed in the cage. When breeding a transgenic positive female mice, they were always placed with a single transgenic negative male mouse, and once again if impregnation did not occur in 3 weeks, the transgenic negative male mouse was replaced. The newborn progeny were screened for the presence of the RIP-CGRP transgene at the age of 2-3 weeks by tail DNA analysis.

3.10 Diabetes Monitoring

Animals were monitored for glycosuria every week using Chemstrip UG (Boehringer Mannheim). If animals were positive in two consecutive urine tests, blood glucose was measured using One Touch blood glucose meter (Lifescan, Inc.). Mice were considered diabetic when their blood glucose levels reached 250 mg/dl.
3.11 Statistical Analysis

Results were analyzed using either the Student \( t \) test or the test for significance of difference between two proportions, when appropriate. To determine the number of animals needed to reach a power of 80\% and an \( a \) level of 0.05 using a two tail test, we performed a power analysis as described elsewhere.
4 RESULTS

4.1 pRIP-hCGRP-Ex2 Construct

As explained in materials and methods following the excision exon 1 and intron 1 from pBS-SK-RIP-hCGRP, pRIP-hCGRP-Ex2 was derived (Figure 2). Both these constructs were subjected to restriction mapping by various endonucleases. The results of these digests are presented in Figure 8 and they show great agreement between the expected and the observed fragment sizes.
4. RESULTS

4.1 BHR-POCEP-E2EP Conversion

An explanation of necessary and important information follows:

and informed that help or information should be provided.

Figure 6: Graph shows conversion of methylation to unmethylated DNA.

Please note: the graph below shows the conversion and the information provided above.

Additionally, the operational parameters were
Figure 8: A: To ensure the proper engineering of the pRIP-hCGRP-Ex2 transgene, the pBS-SK-RIP-hCGRP and pRIP-hCGRP-Ex2 constructs were subjected to restriction mapping as shown below. B: Fragments originating from pBS-SK-RIP-hCGRP and pRIP-hCGRP-Ex2 are bold when differing in size.

<table>
<thead>
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<th>Lane #</th>
<th>DNA Sample</th>
<th>Restriction Enzymes</th>
<th>Expected fragments (kb)</th>
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<tbody>
<tr>
<td>1</td>
<td>1kb Ladder</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Uncut pBS-SK-RIP-hCGRP</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>Uncut pRIP-hCGRP-Ex2</td>
<td></td>
<td>9.5</td>
</tr>
<tr>
<td>4</td>
<td>pBS-SK-RIP-hCGRP</td>
<td>EcoRl &amp; Hind III</td>
<td>3.5, 8.5</td>
</tr>
<tr>
<td>5</td>
<td>pRIP-hCGRP-Ex2</td>
<td>EcoRl &amp; Hind III</td>
<td>3.55, 5.95</td>
</tr>
<tr>
<td>6</td>
<td>pBS-SK-RIP-hCGRP</td>
<td>Xbal &amp; Hind III</td>
<td>0.6, 2.95, 8.5</td>
</tr>
<tr>
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<td>Xbal &amp; Hind III</td>
<td>0.6, 2.95, 5.95</td>
</tr>
<tr>
<td>8</td>
<td>pBS-SK-RIP-hCGRP</td>
<td>BstBI &amp; Hind III</td>
<td>2.5, 3.55, 5.95</td>
</tr>
<tr>
<td>9</td>
<td>pRIP-hCGRP-Ex2</td>
<td>BstBI &amp; Hind III</td>
<td>no 2.5, 3.55, 5.95</td>
</tr>
<tr>
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</tr>
<tr>
<td>11</td>
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<td>XmnI</td>
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<td>EcoRl &amp; BssHI</td>
<td>1.2, 8.3</td>
</tr>
<tr>
<td>16</td>
<td>1kb Ladder</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.2 Beta cells from NOD-CGRP transgenic mice express CGRP

Three NOD-CGRP transgene positive founders were identified by Southern blot analysis of tail DNA (lines 42, 44, 46). Specific expression of the transgene was determined by immunocytochemistry and reverse transcriptase polymerase chain reaction (RT-PCR). Among the three transgene positive lines, two (44 and 46) expressed immunoreactive-CGRP in the pancreas, with mice from line 44 showing the strongest signal. Progeny from line 44 were selected for breeding and analysis. When islets from these NOD-CGRP mice were co-stained with anti-insulin Abs, the signal co-localized with that of CGRP (Fig. 9) indicating that CGRP was produced by beta cells. None of the other tissues examined (brain, lung, kidney, thymus, spleen, intestine, liver, bone marrow) from both NOD-CGRP and NOD littermates expressed detectable level of immunoreactive CGRP. Of note, immunoreactive-CGRP was detected in nerve endings present in most tissues examined, including the pancreas of both NOD-CGRP and NOD littermates. To investigate whether the CGRP transgene was expressed in other tissues, RNA was extracted from organs and isolated islets, and subjected to RT-PCR analysis. As expected PCR products were detected in the pancreas of transgene positive mice as well as in the kidney (Fig 10A), a frequent site of expression of the rat insulin promoter. No expression of RIP-CGRP was seen in tissues other than pancreas and kidney even though we performed 35 cycles of PCR and followed it with Southern analysis. Cultured islets from NOD-CGRP mice, but
not from NOD littermates, expressed RIP-CGRP transcripts at all time points examined (Fig. 10B).
Figure 9: CGRP co-localizes with insulin in islet cells from NOD-CGRP mice. Frozen sections from pancreata of NOD-CGRP mice stained with anti-insulin antibodies (A) and anti-human CGRP (B) reveal the co-localization of insulin and CGRP. Beta cells from NOD mice react with anti-insulin but not with anti-CGRP Ab (D).
Figure 10: Human CGRP is transcribed from the RIP-CGRP transgene. RNA extracted from pancreas, kidney, spleen, liver, lungs, thymus, brain and Langerhans islets of NOD-CGRP and NOD mice was analyzed by RT-PCR and the PCR products subjected to Southern blot hybridization using the RIP-CGRP probe. The predicted 440 bp RT-PCR product from RIP-CGRP mRNA was detected in the pancreas, in the kidney (A) and in the islets cultured for 24, 48 and 72 hours (B) of NOD-CGRP transgenic mice. No signal was detected in transgene negative littermates.
4.3 Beta cells from NOD-CGRP mice produce bioactive CGRP

To investigate whether the transcripts encoded by the RIP-CGRP transgene were expressed by the beta cells as a biologically active peptide, supernatants from isolated islets from NOD-CGRP mice and transgene negative littermates were assayed for CGRP-immunosuppressive activity. Islet supernatants from NOD-CGRP mice inhibited the production of IL-2 by EL-4 cells (Fig. 11). The potency of

Figure 11: Production of biologically active CGRP by islets from transgene positive (A, ■) and transgene-negative littermates (B, ●). One hundred islets per well were cultured in 0.5 ml of culture medium for one to five days. Immunosuppressive activity of the supernatant was assayed using the IL-2-producing mouse T cell lymphoma EL-4. In parallel, EL4.IL-2 cells were cultured in either medium alone ( □ and ○) or supplemented with supernatant derived from islets and previously incubated with the antagonist CGRP 8-37 ( □ and ○). IL-2 concentration in the supernatants was determined by [3H] thymidine incorporation of CTLL cells.
CGRP in these islet supernatants was comparable to that obtained with \(10^{-8}\) M human CGRP (data not shown). Preincubating EL-4 cells with \(10^{-5}\) M of the truncated form of CGRP (CGRP8-37) which acts as a CGRP antagonist\(^6\) prevented the inhibitory effect of the transgenic supernatants suggesting that the effect was indeed mediated by CGRP produced by the transgenic islets. No activity was detected in the islet supernatants from transgene negative littermates. This indicated that beta cells from NOD-CGRP mice produced active CGRP.

4.4 Production of CGRP by beta cells prevents diabetes in male and reduces the incidence in female NOD transgenic mice

The incidence of diabetes in our NOD colony reached 71% in females after 46 weeks of age and 40% in males after 63 weeks and was therefore similar to that reported by other authors\(^8\) (Fig. 12).

While none of the male NOD-CGRP mice developed diabetes, 26% of the female did. The incidence of diabetes was thus reduced by 63% in females aged 52 weeks and older and was significant (\(p<0.05\)) when using a two tail test\(^7\) (Fig. 12). Using the test for significance of difference between two proportions, the \(z\) value was superior to 4 in all groups indicating that the two proportions were significantly different at a 0.001 level.
Figure 12: Incidence of diabetes in NOD-CGRP transgenic mice. Glucosemia was monitored every other week using One-Touch Basic Meter. A reading of 250 mg/dl or more indicated the onset of diabetes.

4.5 NOD-CGRP transgenic mice are immunocompetent

To ensure that CGRP did not have a systemic effect on lymphoid development and function, we performed the following experiments. First, we determined whether T and B cell development was altered in NOD-CGRP mice. As indicated in fig 13A and Table 2, lymphocyte profiles in thymus, spleen and lymph nodes from NOD-CGRP transgenic mice were indistinguishable from those of negative littermates suggesting that CGRP production by beta cells altered neither T and B cell development nor the cellular composition of
Table 2. FACS analysis (in %) of CD4+, CD8+, CD4+CD8+ and Ly5 B220 lymphocytes in lymph nodes, spleens and thymus of 2 NOD control mice and 2 NOD-RIP-CGRP transgenic mice.

<table>
<thead>
<tr>
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<th>NOD</th>
<th>NOD-CGRP</th>
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<tbody>
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<td><strong>CD4+</strong></td>
<td>Lymph nodes</td>
<td>44 / 55</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>34 / 31</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>13 / 13</td>
</tr>
<tr>
<td><strong>CD8+</strong></td>
<td>Lymph nodes</td>
<td>18 / 21</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>14 / 13</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>5 / 4</td>
</tr>
<tr>
<td><strong>CD4+CD8+</strong></td>
<td>Lymph nodes</td>
<td>0.5 / 0.5</td>
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<td></td>
<td>Spleen</td>
<td>0.3 / 0.3</td>
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<td></td>
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<td><strong>B220</strong></td>
<td>Lymph nodes</td>
<td>36 / 22</td>
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<td></td>
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</table>

lymphoid tissues. In addition, T cells responded normally to polyclonal activator such as anti-CD3 and to allogeneic stimulation in vitro, further suggesting that T cell differentiation was not affected by the local production of CGRP (Fig. 13B). We then analyzed whether CGRP could interfere systemically with the priming of naive T cells in lymphoid organs that do not express the transgene. Thus, we analyzed the antigen-specific T cell response to KLH after immunization in vivo with this antigen. As shown in figure 13C, NOD-CGRP mice responded to KLH immunization and the response was similar to that of transgene negative littermates. Thus, in NOD-CGRP mice, lymphoid cells appeared to develop normally and mice
remained immunocompetent. These data indicated that CGRP had no apparent systemic effect, an observation which was corroborated by the low serum concentration of immunoreactive CGRP in NOD-CGRP mice, similar to that of transgene negative littermates (males: 0.49 vs 0.41 ng/ml, females: 0.60 vs 0.62 ng/ml, respectively).
Figure 13: Expression of CGRP by pancreatic beta cells of the NOD-CGRP mice has no detectable systemic effect on the immune response. A: FACS profile of CD4+CD8+, CD4+ and CD8+ T lymphocytes, and B220 B lymphocytes from spleen, lymph nodes and thymus of NOD-CGRP and NOD littermates. B: anti-CD3 activation, allogenic and isogenic stimulation; allogenic stimulation was performed using as APCs spleen cells from CBA/CA (H2k) mice. C: NOD-CGRP and NOD mouse lymphocyte response to KLH immunization. T cell-enriched lymph node cells from NOD (circles) and NOD-CGRP mice (squares) were cultured at increasing density (1, 2 and 4 x 10^5 cells/well, black, grey and white symbols respectively) in Bruff medium supplemented with KLH (0.16 to 100 μg/ml). (squares, NOD-CGRP mice; circles, NOD mice; triangles represent spleen cells from NOD mice (H2k)).
4.6 The production of CGRP by beta cells partially prevents peri-insulitis in NOD-CGRP males

To investigate whether lymphoid cells infiltrated the pancreas of NOD-CGRP males which do not develop diabetes, their pancreata were analyzed histologically at different ages and compared to those from NOD littermates (Table 3). However, due to the late onset of diabetes (30 week) in NOD male, most animals were maintained alive expecting a possible delayed onset of the disease in NOD-CGRP mice. Thus, a limited number of animals were sacrificed for histochemical analysis of the pancreatas.

Table 3. Incidence of peri-insulitis in NOD-CGRP and NOD male mice.

<table>
<thead>
<tr>
<th>Age, (weeks)</th>
<th>Mice developing peri-insulitis, (%)</th>
<th>Islets with peri-insulitis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NOD (n)</td>
<td>NOD-CGRP (n)</td>
</tr>
<tr>
<td>13</td>
<td>100 (3)</td>
<td>25 (4)</td>
</tr>
<tr>
<td>18-20</td>
<td>100 (4)</td>
<td>33 (6)</td>
</tr>
</tbody>
</table>

While all male NOD mice had developed peri-insulitis by the age of 13 weeks, only 25% of NOD-CGRP male mice did so. At that age, only 25% of the islets presented peri-insulitis in both groups. By the age of 18-20 weeks, the percentage of transgene positive males with peri-insulitis increased to 33% while their percentage of islets presenting peri-insulitis remained constant (vs 68% in NOD male). Although the low number of mice observed in each group did not
allow statistical analysis. CGRP expression by beta cells appeared to reduce the incidence of peri-insulitis.

4.7 The infiltrating leukocyte population is similar in NOD-CGRP and NOD littermates

To characterize the pancreatic infiltrate from male NOD-CGRP and NOD mice, pancreata containing insulin-positive cells were subjected to immunocytochemical analysis using anti-CD4, -CD8 and -B220 Abs. The lymphocyte population surrounding the islets of transgenic negative mice during the peri-insulitis stage was composed of a majority of B220+ lymphocytes that represented more than 60% of the total infiltrate. CD4+ cells represented 30% while CD8+ cells represented up to 40% of the total infiltrate. Similar ratios were observed in the infiltrates from transgenic positive littermates in which the percentage of CD4+ and CD8+ cells reached 10-20% and 5-10%, respectively. Comparable ratios of T and B cells surrounding the islets of NOD mice have been reported. These data indicated that the local production of CGRP did not affect the composition of the immune infiltrate present during the pre-diabetic stage with regard to B and CD4/CD8 T lymphocytes.
5 Discussion

The present study demonstrates that the targeted production of the immunosuppressive neuropeptide CGRP to beta cells is sufficient to prevent the onset of diabetes in male and to decrease its incidence in female NOD mice. This prevention is most likely due to a local immunosuppressive effect of CGRP as it is not associated with a generalized immune suppression. Indeed, the average lifespan of transgenic animals was longer than nontransgenic littermates, and comparable normal mouse strains, suggesting that the localized expression of CGRP was not accompanied by undesirable side effects. Although the mechanism by which this immune suppression occurs locally has not been definitively elucidated, islets isolated from our transgenic mice produce active CGRP with a potency estimated to 10^{-8} M CGRP, a concentration that prevents IL-2 and IFN-γ production by Th1 cells in vitro. This suggests that the local concentration of CGRP released by beta cells in vivo may be sufficient to alter the onset of diabetes.

Th1 cells have been shown to accelerate diabetes in NOD mice and are most likely targeted by CGRP in our transgenic mice. CGRP may therefore inhibit the induction or expansion of autoreactive Th1 cells either directly as we reported earlier or indirectly by inhibiting antigen presentation by local antigen presenting cells such as dendritic cells as described in other models. Th1 cytokines are
also thought to be responsible for the activation of other effector cells which can destroy beta cells. In fact CD8+ T cells, NK cells and mediators released by activated macrophages have been implicated in the destruction of beta cells\textsuperscript{9}. We can therefore postulate that CGRP might locally restore an immune balance by either repressing the production of deleterious Th1 cytokines (IL-2, IFN-γ and TNF-α) and/or favoring a Th2 response leading to the production of cytokines such as IL-4 which has been shown to be protective for diabetes\textsuperscript{90}. Furthermore, we cannot rule out that CGRP may act directly on other effector cells such as NK\textsuperscript{91} or macrophages\textsuperscript{92}. Dr. Vignery previously has demonstrated that macrophages express functional receptors for CGRP\textsuperscript{93} and more recently that CGRP down regulates the production of TNF-α (Millet and Vignery, unpublished data). Additional characterization of the cells infiltrating the islets and determination of their cytokines produced in NOD-CGRP mice are required to address these issues.

Importantly, although NOD-CGRP males do not develop the disease, they still exhibit a perivascular and peri-islet accumulation of lymphoid cells. While the proportion of islets surrounded by lymphoid cells appears lowered, the composition of the infiltrates does not appear modified by the expression of the transgene. If confirmed by further investigations, this would suggest that CGRP
might protect from diabetes by preventing the infiltration and clonal expansion of immune cells around the islets.

Thus the local expression of the immunosuppressive peptide CGRP by beta cells prevents their destruction and therefore diabetes in NOD mice. This observation opens possibilities to treat or prevent diabetes by engrafting islets expressing CGRP into NOD mice.

From the concept that Th1 cells contribute to the pathogenesis of several organ-specific autoimmune diseases, targeted immuno intervention aiming to restore an immune imbalance based on a cell-specific transgene expression of an immunosuppressive peptide appears as a promising approach. Similar targeted gene therapy can be applied to other organ-specific autoimmune diseases such as collagen-induced arthritis, Hashimoto’s thyroiditis, Grave’s disease or to conditions in which immune cells are believed to be pathogenic mediators.

Finally, an alternative and somewhat unlikely possibility which has not been disproven by our studies is that the transgene effect seen can be the result of the transgene having randomly inserted into, and thus disrupting the function of, a diabetes susceptibility gene. Since such susceptibility genes are mainly recessive, inactivating one allele would make the animal diabetes resistant. However improbable this and perhaps other explanations may seem, inherently uncertain nature of science forces us to humbly accept the fact that if
some event is improbable, that does not necessarily mean that it is impossible.

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