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Immunotherapy of the Nonobese Diabetic Mouse: Treatment with an Antibody to T-Helper Lymphocytes

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Spontaneous diabetes mellitus was blocked in nonobese diabetic mice by treatment with a monoclonal antibody against the L3T4 determinant present on the surface of T-helper lymphocytes. Sustained treatment with the monoclonal antibody led to cessation of the lymphocytic infiltration associated with the destruction of the insulin-producing β cells. Moreover, the mice remained normoglycemic after the antibody therapy was stopped. These studies indicate that immunotherapy with monoclonal antibodies to the lymphocyte subset may not only halt the progression of diabetes, but may lead to long-term reversal of the disease after therapy has ended.

THERE IS INCREASING EVIDENCE that human insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease and that IDDM results from immune destruction of the insulin-producing β cells normally found in the islets of Langerhans (*1*). Nonobese diabetic (NOD) mice spontaneously develop diabetes (*2*) resembling human IDDM. As in human IDDM, the NOD mice have progressive lymphocytic infiltration into the islets (insulitis) before the expression of overt diabetes (*2–4*), and cytoplasmic antibodies to islet cells appear in their serum during the development of insulitis (*4, 5*). Susceptibility to diabetes in both humans and NOD mice is strongly associated with genes of the major histocompatibility complex (MHC) (*6*). Overt diabetes is characterized by polyuria, polydipsia, hyperglycemia, and glycosuria, and NOD mice develop acute ketoacidosis, which is fatal unless the mice are treated with insulin (*2, 7*).

The specific immunologic pathways and cell types responsible for islet cell destruction in NOD mice are not clearly delineated (*8*). However, recent studies suggest that the T lymphocyte subset that expresses the L3T4 surface marker is important in the pathogenesis of the disease (*9*). T lymphocytes of the L3T4 phenotype are a distinct subpopulation of mature T cells that function as helper-inducer cells in the activation of both humoral and cellular immunity (*10*). The L3T4 lymphocyte subset is responsible for MHC class II–restricted antigen recognition on antigen-presenting cells (*11*); the human homolog to the murine $L3T4^+$ T cell is the $CD4^+$ T cell (*11*). We have been able to block the progression and subsequent expression of overt diabetes in NOD mice by a course of treatment with a monoclonal antibody to L3T4. Such an approach may be feasible for treatment of patients with subclinical manifestations of IDDM, since we show that antibody therapy initiated late in disease progression was effective in reversing the advanced phases of islet cell destruction. Moreover, upon cessation of therapy the mice have remained disease-free without further treatment.

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The monoclonal antibody used in these studies, GK1.5, is a cell-depleting antibody. When administered to mice at doses greater than 300 μg, this antibody causes sustained reduction of more than 90% of the circulating $L3T4^+$ cells (*12*). GK1.5 has been successfully used in vivo as an immunotherapeutic agent to treat other experimental and spontaneous autoimmune diseases, including systemic lupus erythematosus (*13*), experimental allergic encephalomyelitis (*14*), and type II collagen-induced arthritis (*15*). In addition, a single course of this antibody has been shown to allow indefinite acceptance of transplanted allogeneic murine islets of Langerhans (*16*). GK1.5 and other antibodies to L3T4 are particularly suitable for serotherapy, since these reagents can suppress the humoral immune response (*12, 17*) and induce tolerance to select protein antigens, including the monoclonal antibody to L3T4 itself (*17*).

When NOD mice are 30 to 50 days old, mononuclear cells begin to infiltrate the perivascular and periductal areas around the

Table 1. Prevention of diabetes in NOD mice by long-term treatment with GK1.5. Rat monoclonal antibody GK1.5 (immunoglobulin G2b) to mouse L3T4, purified from ascites fluid, was administered intraperitoneally to 90- to 110-day-old NOD female mice. Incidence of diabetes is shown as the ratio of the number of diabetic mice to total number of mice in the group at 260 days of age.

Amount of GK1.5 administered (μg)	Incidence of diabetes	Time of onset of diabetes (days)
600	18/21	157 ± 33
600, then 100 weekly	2/25	156 ± 43
None	29/35	173 ± 42

islets (*2, 7, 18*). Inflammation continues until mice are 80 to 100 days old at which time the islets are massively infiltrated (*18*). Histochemical analysis at age 40 to 90 days (*18*) shows that in the early stage of insulitis most of the infiltrating cells are Lyt-1^+ ($L3T4^+$ cells are Lyt-1^+, Lyt-2^-); however, as the lesions progress, cells bearing surface immunoglobulin (B lymphocytes) predominate (*7, 18*). Coincident with the expression of insulitis, there is a decline in pancreatic insulin concentration (*7*). At the onset of overt hyperglycemia (diabetes), usually at age 150 to 200 days, approximately 90% of the insulin-secreting β cells have been destroyed (*7, 19*). The incidence of spontaneous hyperglycemia is related to gender; 70 to 90% of the female NOD mice and fewer than 20% of the male NOD mice become hyperglycemic (*1, 2*).

To test the effectiveness of anti-L3T4 immunotherapy during disease progression, we used litter-matched female NOD mice aged 90 to 110 days (well into the late stage of advanced insulitis). Mice were randomly assigned to three experimental groups. Group 1 received 600 μg of GK1.5 intraperitoneally (200 μg/day for 3 days), with no further treatment for the duration of the experiment. Group 2 received the same initial 600 μg of antibody but was also given maintenance weekly injections (100 μg). Group 3 was untreated for the duration of the experiment. Plasma glucose values were used to monitor the development of hyperglycemia. Diabetes was diagnosed when blood glucose values were greater than 500 mg/dl.

The frequency and mean onset time of diabetes in control and experimental animals is shown in Table 1. By 260 days of age, approximately 80% of the untreated NOD females had diabetes. Animals receiving only a single course of antibody therapy had an incidence of hyperglycemia comparable to that in untreated controls. Unlike the controls and the mice treated for a short term, 23 of the 25 mice receiving long-term immunotherapy did not become hyperglycemic. Littermates of the two mice that developed hyperglycemia under long-term antibody treatment had accelerated onset of diabetes (123 ± 9 days, $n = 4$; and 122 ± 8 days, $n = 3$). It is likely that at the initiation of therapy these animals already had a reduced β cell mass, and thus antibody treatment was ineffective in preserving sufficient insulin secretory function to maintain normoglycemia.

To determine the effects of antibody administration on insulitis, we monitored lymphocytic infiltration into the islets of Langerhans by histologic examination. Sections stained with hematoxylin and eosin were scored for islet lymphocytic infiltration by a pathologist who was not aware of the treatment status of the mice. Randomly selected control NOD female mice older than 90 days had heavy lymphocytic infiltration of their islets (Fig. 1A). Continued examination of control animals and mice treated with a single course of antibody had uninterrupted islet infiltration. When hyperglycemia developed, these mice had abnormal islet morphology as well as greatly reduced islet size and numbers. In contrast, mice receiving weekly GK1.5 injections had clearing of islet mononuclear cell infiltration and normal appearing islets after 90 days of therapy (Fig. 1B). Long-term antibody treatment did not cause immediate depletion of lymphocytes from islet tissue, however. Significant islet infiltration was noted as late as 1 month after the initiation of long-term treatment, and no histologic change was seen in any of the mice given a short course of antibody.

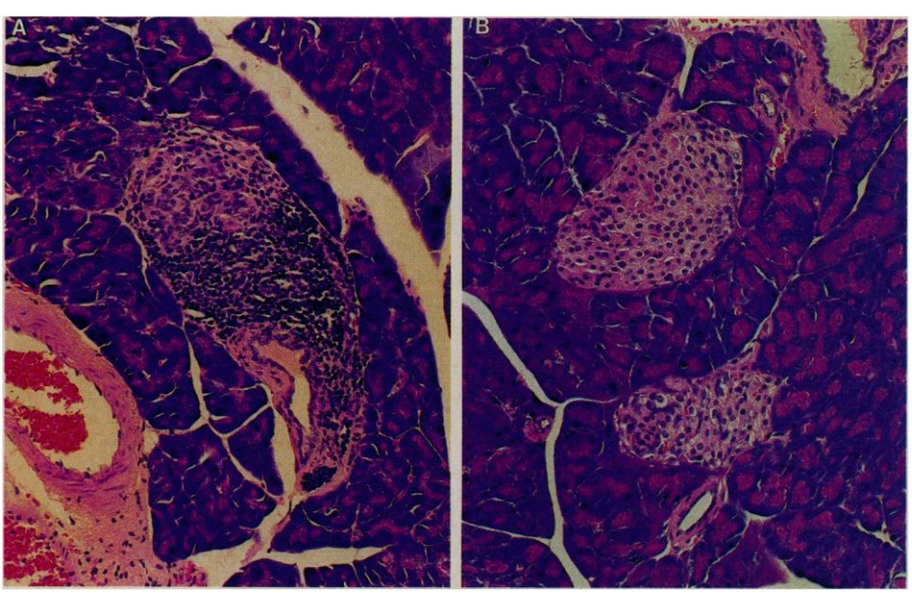

Fig. 1. Effect of long-term therapy with antibody to L3T4 on lymphocytic infiltration into pancreatic islets. (**A**) Islet from an untreated 110-day-old NOD female mouse; (**B**) islet from a 180-day-old NOD female mouse treated for 91 days with weekly injections of GK1.5 (×400).

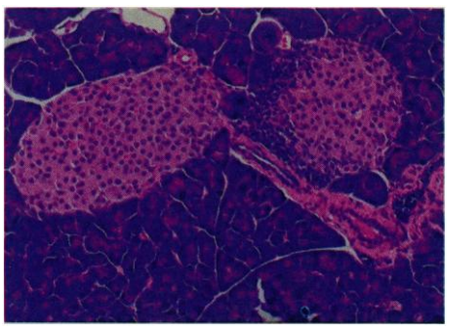

Fig. 2. Reinfiltration of islets in an NOD female mouse 46 days after withdrawal of weekly injections (15 injections total) of GK1.5 treatment (×320).

The possibility that progression to disease was not simply blocked but, in fact, reversed, by long-term treatment with antibody to L3T4 was examined by withdrawing therapy after approximately 100 and 150 days of treatment. The mice were bled at 2-week intervals to determine the time of persistent normoglycemia (plasma glucose below 250 mg/dl) after cessation of therapy. Of 18 mice treated, one became diabetic. In that mouse, hyperglycemia occurred approximately 1 month after therapy withdrawal. Six mice selected at random for histologic study were normoglycemic at the time they were killed. The other eleven animals have maintained normal blood sugar levels for more than 225 days after cessation of therapy. Despite the absence of diabetes in these mice, histologic analysis of pancreases of mice from this group revealed the recurrence of insulitis. Recurrent mononuclear cell infiltration was observed as early as 14 days after cessation of therapy, and pancreatic tissue from all mice off therapy for more than 30 days showed focal lymphocytic infiltrates in some islets beginning at one pole. After 30 days, however, the severity of inflammation, as judged by the numbers of islets involved and penetration of lymphocytes into the islets, did not appear to increase (Fig. 2). This demonstration of insulitis without expression of overt hyperglycemia is not unprecedented. Although diabetes in NOD mice is always preceded by severe insulitis, there are several examples of NOD mice (especially males) (*2*) or F_1 mice with a NOD mouse as one parent (*20*) remaining normoglycemic despite insulitis. Genetic studies reveal that insulitis and the

development of overt hyperglycemia are controlled by distinct but overlapping genetic loci (*20, 21*).

A current hypothesis suggests that islet inflammation predisposes an animal to the development of diabetes but that other elements of the immune system determine progression to overt disease (*19, 21*). Since treatment with the antibody to L3T4 caused immediate and profound inversion of the ratio of $L3T4^+$ to $Lyt\text{-}2^+$ (inducer to suppressor) T cell populations, we studied the reestablishment of these subsets in the normoglycemic animals treated long-term with GK1.5. Repopulation of circulating $L3T4^+$ and $Lyt\text{-}2^+$ cells was monitored by fluorescence-activated cell sorter (FACS) analysis. Control, age-matched ICR mice (the non-

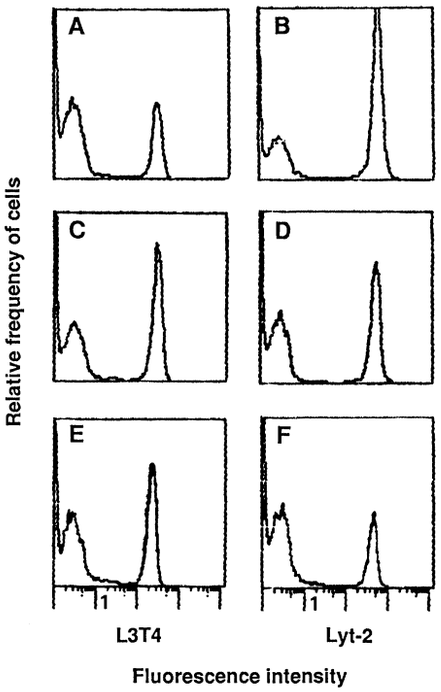

Fig. 3. Recovery of the T lymphocyte subset ratio after cessation of long-term therapy with antibody to L3T4. Fluorescence analysis of purified peripheral blood lymphocytes from mice at (**A** and **B**) 30 days, (**C** and **D**) 84 days, and (**E** and **F**) 150 days after the last injection of 15 weeks of treatment with GK1.5. Ficoll-purified lymphocytes were incubated for 30 minutes at 4°C with previously titered GK1.5 (left) or YTS.169.4 (a monoclonal antibody to Lyt-2) and then washed twice. Fluorescein-conjugated goat antibody to rat immunoglobulin (absorbed with mouse immunoglobulin, Caltag) was added as a second-step reagent for 30 minutes at 4°C and then washed once. Dead cells were excluded from analysis by the scatter-gating method and by propidium iodide staining. Samples were analyzed on a FACS II system (Becton Dickinson) equipped with logarithmic amplifiers. Data on the x axis are expressed on a $\log_{10}$ scale of increasing fluorescence intensity and on the y axis as relative cell numbers in arbitrary units. The ratio of $L3T4^+$ to $Lyt\text{-}2^+$ cells was: 30 days (30% to 70%), 84 days (52% to 48%), and 150 days (60% to 40%).

inbred parental strain to NOD mice) or untreated diabetic NOD mice have approximately 60 to 70% $L3T4^+$ cells and 30 to 40% $Lyt\text{-}2^+$ cells in their peripheral blood (*22*). Treatment with GK1.5 selectively reduces $L3T4^+$ cells from peripheral blood, lymph nodes, and spleen (*12, 13–15*). When NOD mice were withdrawn from long-term GK1.5 therapy, a prolonged inversion of the $L3T4^+/Lyt\text{-}2^+$ ratio occurred (Fig. 3). However, 150 days after termination of therapy these animals had normal relative T cell subset frequencies (Fig. 3, E and F). Thus, NOD mice given long-term treatment with the antibody to L3T4 had a slow recovery of peripheral $L3T4^+$ T-helper cells and persistent normoglycemia, despite rapid reinfiltration of islets after therapy ended.

Related studies with cyclosporin A (CsA) as the immunosuppressant in the diabetes-prone BB rat model demonstrate that, under defined conditions (dose, duration, and timing of therapy), CsA can provide complete protection from diabetes (*23*). However, maximum results were achieved only when therapy was initiated before the expression of insulitis. We similarly found that dose and length of therapy are crucial to the efficacy of anti-L3T4 treatment in NOD mice. But, unlike the situation in the BB rat, effective antibody treatment in NOD mice can be initiated during the advanced stages of islet pathogenesis. One difference between the two models is the time course of islet destruction. The time from peri-islet infiltration to glycosuria in BB rats is approximately 7 to 21 days (*23*) in contrast to the 100 to 150 days of infiltration preceding hyperglycemia in NOD mice (*2*).

Long-standing normoglycemia after cessation of long-term therapy suggests that antibody treatment protected the islets from further destruction. These results were unexpected, since therapy was initiated in mice undergoing active immunity against islet β cells. Although antibodies to L3T4 induce tolerance to protein antigens (*17*), such tolerance occurs only when the initial exposure to antigen follows antibody treatment (*12*). Mice that are primed with antigens before short-term treatment with antibody to L3T4 can, after a transient delay, mount antigen-specific humoral responses (*12, 24*). It is possible that sustained reduction of $L3T4^+$ T cells in conjunction with long-term exposure to the diabetogenic antigen created an environment that allowed dominant suppressive mechanisms to emerge. An alternative explanation is that the diabetogenic self-antigen or a cross-reactive antigen may have appeared in NOD mice only transiently during development and disappeared during the course of therapy. Since the levels of the $L3T4^+$ T cell subset returned to normal without concomitant disease, the sparing of islet function due to blanket immunosuppression by the anti-L3T4 treatment is unlikely. Further adoptive transfer and transplantation studies should illuminate the mechanisms underlying the reversal of disease by long-term anti-L3T4 therapy.

How the immune system selectively destroys islet β cells in IDDM is still unknown. Our findings demonstrate that the L3T4 subset is essential throughout the course of disease pathogenesis in NOD mice regardless of the specific effector mechanisms involved. What is more important, these studies point to the possibility of an immunotherapy for IDDM that not only halts progression of disease while therapy is given but may result in long-term reversal of disease after therapy is ended. The implications for treatment in human IDDM are obvious, and these studies emphasize the need for appropriate immunotherapy early in disease progression in order to preserve a sufficient functional β cell mass to maintain normoglycemia. Thus, by monitoring genetic predisposition to the development of diabetes in the appropriate high-risk populations, it may be possible to design rational and successful immunotherapeutic strategies for controlling IDDM.

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Aggregation of Lysine-Containing Zeins into Protein Bodies in *Xenopus* Oocytes

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Zeins, the storage proteins of maize, are totally lacking in the essential amino acids lysine and tryptophan. Lysine codons and lysine- and tryptophan-encoding oligonucleotides were introduced at several positions into a 19-kilodalton zein complementary DNA by oligonucleotide-mediated mutagenesis. A 450–base pair open reading frame from a simian virus 40 (SV40) coat protein was also engineered into the zein coding region. Messenger RNAs for the modified zeins were synthesized in vitro with an SP6 RNA polymerase system and injected into *Xenopus laevis* oocytes. The modifications did not affect the translation, signal peptide cleavage, or stability of the zeins. The ability of the modified zeins to assemble into structures similar to maize protein bodies was assayed by two criteria: assembly into membrane-bound vesicles resistant to exogenously added protease, and ability to self-aggregate into dense structures. All of the modified zeins were membrane-bound; only the one containing a 17-kilodalton SV40 protein fragment was unable to aggregate. These findings suggest that it may be possible to create high-lysine corn by genetic engineering.

THE SEED STORAGE PROTEINS OF CEREALS, prolamines, are noted for their high content of proline and glutamine and the virtual absence of lysine. Since prolamines account for approximately half of the total seed protein, cereals are generally deficient in lysine. Previously, the only means by which this essential amino acid could be increased was by selecting strains with low prolamine content (*1*); however, the softer kernels and smaller yields of such strains have limited their usefulness (*2*). Since genes encoding storage proteins with higher lysine contents do not normally exist in cereals, an alternative to conventional plant breeding is to modify existing genes in vitro by genetic engineering and to express them in transgenic plants (*3*).

The prolamines of corn, *Zea mays* L., are known as zeins (*4*). These proteins perform no known enzymatic function and thus appear to be ideal candidates for amino acid modification by genetic engineering. The three types of zeins (α, β, and γ) (*5*) are synthesized on rough endoplasmic reticulum and aggregate within this membrane in dense deposits called protein bodies. The mechanisms responsible for protein body formation are thought to involve hydrophobic and weak polar interactions between zeins (*6*). The absence of lysine in these proteins may reflect the fact that charged amino acids would adversely affect protein aggregation and thus the formation of normal protein bodies.

Earlier studies (*7–9*) demonstrated that injection of zein messenger RNA (mRNA) into *Xenopus* oocytes results in the synthesis and processing of zein proteins into membrane-enclosed structures with the physical characteristics of protein bodies from maize. With this system it is possible to study the consequences of lysine substitutions and other modifications to zein proteins on their ability to form protein bodies. We have assayed two properties relating to the assembly of modified zeins into protein bodies in oocytes: (i) transport into membrane-bound structures and (ii) aggregation into vesicles sedimenting with a high density. Surprisingly, we find that only the most severe modification of a zein protein hinders its ability to form protein bodies.

Figure 1 shows the amino acid sequence of a 19-kD α zein arranged in a manner that corresponds to the structural domains proposed by Argos *et al.* (*6*); the indicated protein changes were made from three types of modifications to the corresponding complementary DNA (cDNA) clone. Single and double amino acid substitutions incorporate lysines into several positions in place of neutral amino acids; oligopeptide insertions place lysine- and tryptophan-rich peptides within the zein molecule; and the large peptide insertion, pMZ44-SV40 (simian virus 40), places 17 kD of an unrelated, hydrophilic protein (from the SV40 VP2 protein) into the NH_2-terminal region.

In order to synthesize the modified zeins and assay their ability to form protein bodies, we produced artificial mRNAs for them in vitro by using SP6 RNA polymerase. These mRNAs were injected into *Xenopus* oocytes, and the proteins were labeled by subsequent injection of [^{3}H]leucine. After fractionation, the zeins were extracted and subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography. Amino acid–substituted zeins were detected, and their molecular size was the same (migration in SDS-PAGE) as that of the unmodified protein. The signal peptides of zeins are correctly removed in *Xenopus* oocytes (*7*), and this is also true of the single and double lysine-substituted zeins. The actual incorporation of lysines into the modified zeins was independently confirmed by isoelectric focusing (IEF) (*10*). We conclude that the introduction of lysine into various positions in the molecule does not significantly affect its synthesis, stability, or the cleavage of its signal peptide. Injection of RNAs for peptide insertion mutants and the pMZ44-SV40 zein similarly resulted in production of stable proteins of the expected molecular size.

In that the modified zeins were faithfully synthesized and processed in oocytes, we next examined their assembly into protein bodies. Hurkman *et al.* (*8*) showed that total zein mRNA injected into oocytes can direct the synthesis and assembly of zeins into dense, membrane-bound structures similar to maize protein bodies.

The appearance of zein in membrane-bound organelles was assayed by the protein's resistance to proteolytic digestion. A wild-type 19-kD zein was synthesized in oocytes by injection of synthetic mRNA, and the homogenized eggs were treated with protease K. The zein was resistant to digestion with protease (up to 1 mg/ml) unless 1% Triton X-100 was present, in which case it was completely digested (Fig. 2A). To demonstrate that this resistance is the result of internalization within membranous structures and not merely a consequence of hydrophobic interactions with membranes, we performed the following experiment (Fig. 2B). Purified, labeled zein

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