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34. Recombinant C*AAX* farnesyltransferase was produced in a baculovirus expression system (Y. Reiss, M S Brown, J L. Goldstein, in preparation). Briefly, the cDNAs encoding the α and β subunits of rat C*AAX* farnesyltransferase were each excised from their parent vectors (*31*) and inserted separately into the corresponding sites of the baculovirus expression vector pVL1393 (*32*). Recombinant baculoviruses encoding both subunits were introduced together into Sf9 cells. Seventy-two hours after infection, the cells were harvested and disrupted in a Parr cell disruption bomb C*AAX* farnesyltransferase was isolated by chromatography on Q-Sepharose. The enzyme was judged to be ~90% pure by Coomassie blue staining after SDS PAGE.
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40. Each cell monolayer was harvested by rinsing three times with 3 ml of 50 mM tris-HCl (pH 7.5), 0.15 M NaCl. Lysis buffer (300 μl) [0.5× Dulbecco's phosphate-buffered saline containing 1% Triton X-100, leupeptin (5 μg/ml), pepstatin (5 μg/ml), 0.5 mM phenylmethylsulfonyl fluoride, and 0.05 trypsin inhibitor units per milliliter of aprotinin] was added to each monolayer. After incubation on ice for 5 min, the lysates were centrifuged for 30 s in a microfuge at 12,000*g*. The resulting supernatant was transferred to a new tube, and each sedimented sample was resuspended in lysis buffer (60 μl). Protein concentrations were determined by the BCA protein assay reagent (Pierce, Rockford, IL) according to the manufacturer's directions. Samples were mixed with 2× SDS sample buffer (*35*) and heated at 95°C for 5 min before electrophoresis.
41. Portions of the Triton X-100–soluble fraction (300 μg of protein) were incubated with a rat monoclonal antibody to Ras (1 μg) (Oncogene Science, Inc.) overnight at 4°C on a rotating platform. Immune complexes were precipitated by addition of 25 μl of protein A–agarose suspension that had been precoated with goat antibody to rat immunoglobulin G (Oncogene Science, Inc.) according to the manufacturer's directions. After a 30-min incubation at 4°C, the agarose beads were centrifuged and washed five times with 1 ml each of washing solution [50 mM tris-chloride (pH 7.5), 50 mM NaCl, 0.5% (w/v) deoxycholate, 0.5% (v/v) Nonidet P-40, and 0.1% (w/v) SDS]. Each sample received 75 μl of 1× SDS sample buffer (*35*) and was heated for 5 min at 95°C before electrophoresis.
42. The H-Ras–transformed cells were generated by transfection of Rat1 fibroblasts with an SV40-driven expression vector containing an *H-ras* gene in which glycine at amino acid 12 was replaced by valine [P. H. Seeburg, W. W. Colby, D. J. Capon, D. V. Goeddel, A. D. Levinson, *Nature* **312**, 71 (1984)]. Cells that overgrew the monolayer were extracted and plated into agar to obtain a cell line displaying a fully transformed phenotype. The Src-transformed cells were generated by transfection of Rat1 fibroblasts with a vector containing the v-*src* gene [M. A. Snyder, J. M. Bishop, W. W. Colby, A. D. Levinson, *Cell* **32**, 891 (1983)] as well as the *neo* gene that confers G418 resistance, both under control of SV40 early promoters. G418-resistant cell clones that displayed a transformed phenotype were used to generate a cell line.
43. We thank D. Noble-Morgan, T. Martin, S. Broz, D. Burdick, D. Oare, K. Paris, and M. Reynolds for technical assistance. Supported by NIH grant HL 20948, by a grant from The Perot Family Foundation, and by a postdoctoral fellowship from the Helen Hay Whitney Foundation (G.L.J.).

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Induction of Type I Diabetes by Interferon-α in Transgenic Mice

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Type I diabetes is an autoimmune disease involving an interaction between an epigenetic event (possibly a viral infection), the pancreatic β cells, and the immune system in a genetically susceptible host. The possibility that the type I interferons could mediate this interaction was tested with transgenic mice in which the insulin-producing β cells expressed an interferon-α. These mice developed a hypoinsulinemic diabetes associated with a mixed inflammation centered on the islets. The inflammation and the diabetes were prevented with a neutralizing antibody to the interferon-α. Thus, the expression of interferon-α by the β cells could be causal in the development of type I diabetes, which suggests a therapeutic approach to this disease.

Type I diabetes is caused by the progressive loss of pancreatic β cells and is associated with several autoimmune phenomena (*1*). Although particular alleles closely linked to the major histocompatibility complex (MHC) class II locus increase the risk of developing type I diabetes (*2*), studies of identical twins have implicated poorly characterized environmental factors in the initiation of the disease (*3*). Several reports have suggested that viral infections could be these environmental agents in type I diabetes (*4*). However, many different viruses are epidemiologically linked to diabe-

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tes, and at diagnosis there is usually no active viral infection. Expression of interferon-α (IFN-α) is induced in epithelial cells by several different viruses (*5*). Although named for its antiviral activities, IFN-α also affects natural killer (NK) cells (*6*, *7*) and macrophages (*8*) and modifies the antibody response (*9*, *10*). Because IFN-α can provide a link between the environment (a viral infection) and an immune response, we have investigated the possibility that it could initiate an autoimmune response capable of destroying the β cells. We tested this possibility by developing a transgenic mouse model in which the β cells express an active IFN-α. We show that IFN-α expressed in the islets can induce a syndrome resembling type I diabetes, and we present the results of preventive therapy using a neutralizing antibody to IFN-α (anti–IFN-α).

We generated four transgenic mice (*11*) that carry a transgene containing the regulatory region of the human insulin gene, a cDNA that encodes a hybrid human IFN-α (A/D Bgl II) that unlike most human IFN-α's is active on mouse cells (*7*, *12*), and the polyadenylate addition signal from the hepatitis surface antigen gene. This general form of a transgene can efficiently drive expression of a heterologous gene in the β cells (*11*, *13*). One line of transgenic mice derived from one of these founders, referred to here as Iia (insulin IFN-α), is presented in detail. Of the other three founder transgenic mice, one did not transmit the transgene and so was not further analyzed, one was detected as being diabetic (glucose > 400 mg/dl) at around 10 weeks of age but died without transmitting the transgene, and the third has led to the generation of a line of transgenic mice that have a qualitatively similar but less severe form of the insulitis described below and have an incidence of diabetes of ~10% (*14*).

The Iia founder mouse was backcrossed to both outbred albino mice (CD1) and to inbred C57BL/6J mice. The Iia(CD1) transgenic mice became diabetic with a cumulative incidence of greater than 50% (Fig. 1A) and a median time to onset of about 4 weeks. The incidence in the Iia(C57BL/6) mice was less than 5% (*15*). In the Iia(CD1) transgenic mice, diabetes onset was detected from 2 weeks to 6 months of age (Fig. 1B). The diabetes was characterized as a persistent hyperglycemia with polyuria and polydipsia. In the diabetic mice the glucose concentrations were maintained between 250 and 400 mg/dl for up to 6 weeks before glucose concentrations further increased and the animals died, apparently as a consequence of the hyperglycemia. The diabetic mice were severely hypoinsulinemic (Fig. 1C). Thus, the consequence of the expression of the IFN-α transgene was a hypoinsulinemic diabetes.

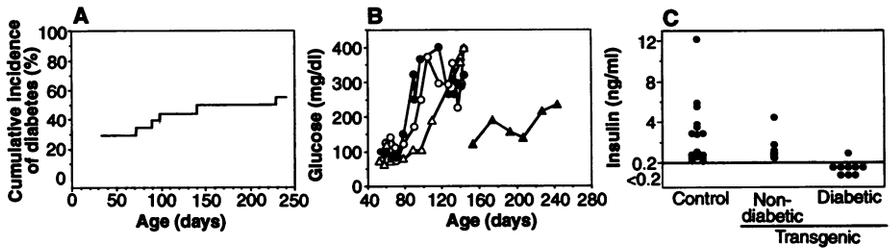

Fig. 1. Transgenic mice in which the β cells express IFN-α developed a hypoinsulinemic diabetes. (**A**) Life table analysis of 20 male transgenic mice. (**B**) Glucose concentrations as a function of age for a representative group of mice. The mean glucose concentration in nontransgenic mice was ~110 mg/dl. (**C**) Insulin concentrations (nanograms per milliliter of serum) in nontransgenic mice and in transgenic (diabetic and nondiabetic) mice. The results were derived from a pool of litters obtained from the N3 backcross to CD1 mice. We monitored the mice at the indicated ages for glucose by analyzing venous blood with a Lifescan glucose analyzer. Insulin concentrations in sera were determined in nonfasted animals by established radioimmunoassay (*32*).

In pancreata from humans with type I diabetes, the early lesions are characterized by a mononuclear cell infiltrate into the islets, many of which still contain β cells with immunoreactive insulin, and a minor infiltration into the surrounding acinar tissue (*1*). Although human diabetics lose almost all of their β cells, the absolute number of islet cells that stain for glucagon or somatostatin is relatively unchanged.

In young (4- to 6-week) diabetic Iia-(CD1) transgenic mice, mononuclear cells infiltrated around and within the islets (Fig. 2). This insulitis was accompanied by β cell necrosis (*16*). In transgenic mice that had been diabetic for two or more months, the inflammation became less marked and left islets that were shrunken and relatively devoid of histologically obvious β cells (Fig. 2). In young diabetic mice a significant number of cells within the islets contained insulin regardless of whether these islets had a lymphocytic involvement, whereas mice that had been diabetic for more than 6 weeks had fewer insulin-containing cells and a relative increase in the number of both somatostatin- and glucagon-containing cells (Fig. 3).

By immunocytochemistry, the mononuclear cells infiltrating the islets in young transgenic mice included CD4-positive lymphocytes. The section shown in Fig. 4C from the pancreas of a transgenic mouse shows an infiltrated islet comparable to those shown in Fig. 2, C and D, and the presence of CD4-positive lymphocytes. To obtain an estimate of the ratio of CD4 to CD8 cells that was not biased by intrapancreatic regional differences, we perfused pancreata from control and transgenic mice with phosphate-buffered saline (PBS), labeled single-cell preparations with monoclonal antibodies (mAbs), and analyzed the

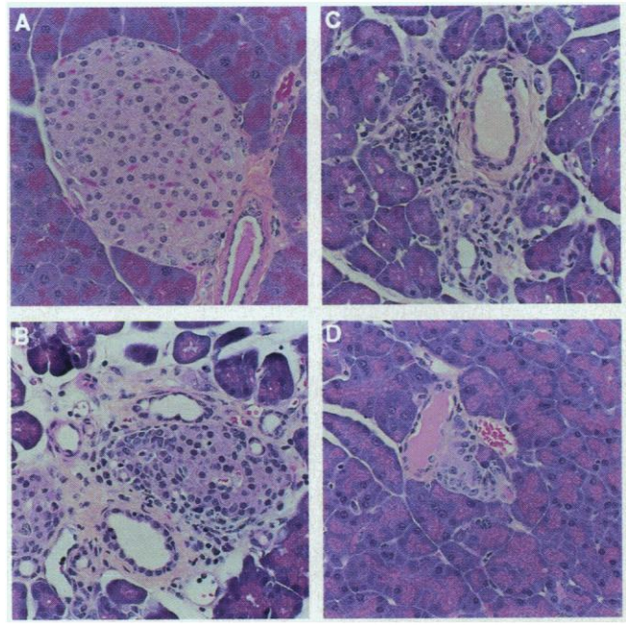

Fig. 2. The development of diabetes is coincident with an invasion of leukocytes into the islets. An islet from a normal mouse (**A**) appears rounded, with the majority of the cells intact and well granulated. In contrast, the islet from a young transgenic mouse (age 6 weeks, diabetic at 2 weeks) has a severe mixed leukocytic inflammation invading the islet (**B** and **C**). In mice that have been diabetic for 3 months, the islets appear shrunken and atrophied (**D**). Pancreata were removed from the indicated mice and fixed in 10% formalin, and 5-μm sections were stained with hematoxylin and eosin. Magnification, ×350.

cells by flow cytofluorometry. Pancreata from control mice had a small residual contaminating lymphocyte population (CD4, 1 to 3% and CD8, 0.1%), demonstrating that the pancreata were not significantly contaminated with lymph nodes. In contrast, in the pancreata from the diabetic mice, 14.1% of the cells stained positive for CD4 and 3.1% for CD8 (Fig. 4). The relative order of appearance of monocytes, lymphocytes, and NK cells is not yet known, nor is it clear which of these cell types are responsible for the β cell necrosis.

Because diabetes occurred in two independent lines of mice (in addition to a third founder mouse that became diabetic but died without transmitting the transgene), the pathology was probably a result of the presence and expression [IFN-α can be detected by immunocytochemistry (*14*)] of the transgene rather than an insertional event. To demonstrate that the disease is dependent on secretion of the biologically active protein, we undertook a prophylactic intervention with a mAb that neutralizes the biological activity of the human IFN-α synthesized by these transgenic mice. Beginning at 2 weeks of age and continuing through 6 weeks, transgenic mice were treated three times per week with a neutralizing mAb or an isotype-matched control (Table 1). After 4 weeks of treatment there were significantly fewer ($P < 0.01$) diabetic mice in the group receiving the mAb to IFN-α (anti–IFN-α) as compared with the group of mice receiving the isotype-matched control. At the end of the 4-week treatment, the pancreata were examined histologically in a blinded manner (Table 1). The inflammation associated with the islets was graded on a scale from 0 (minimal) to 5 (severe), and the intensity of staining of immunoreactive insulin within the β cells was graded from 0 (minimal) to 6 (very intense). The mean histological score for the insulitis in the transgenic mice treated with the control mAb was 3.2, whereas the score was 1.5 for the mice receiving 4 weeks of treatment with the anti–IFN-α. The mean insulin score was 3.1 in the control group and 5.1 in the treated group. In addition, we observed that the lesions present in the exocrine pancreas were less severe in the group treated with anti–IFN-α than in the group treated with the control mAb (*14*).

We have demonstrated that the expression of IFN-α in the β cells of a transgenic mouse can lead to a hypoinsulinemic diabetes. The loss of the β cells in the diabetic mice was selective in that there was a relative sparing of the α and δ cells and the β cell loss was associated with a mixed inflammation centered on the islets. Because this mouse model reproduces the IFN-α expression seen in human diabetics (*17*) and leads to a pathology that has many similarities to human type I diabetes (*1*), expression of IFN-α by islet cells may be a causal factor in the development of this disease in humans. That comparable transgenic mice expressing interleukin-2 (IL-2) (*18*), tumor necrosis factor–α (TNF-α) (*19*), or TNF-β (*20*) do not develop diabetes emphasizes the relevance of the results reported here. A causal relation would be supported by reports of autoimmune diseases being induced in patients treated with recombinant IFN-α (*10*, *21*).

The possibility that the activation of local macrophages (*8*) and NK cells (*6*, *7*) by IFN-α is a primary cause of the β cell loss would be consistent with reports demon-

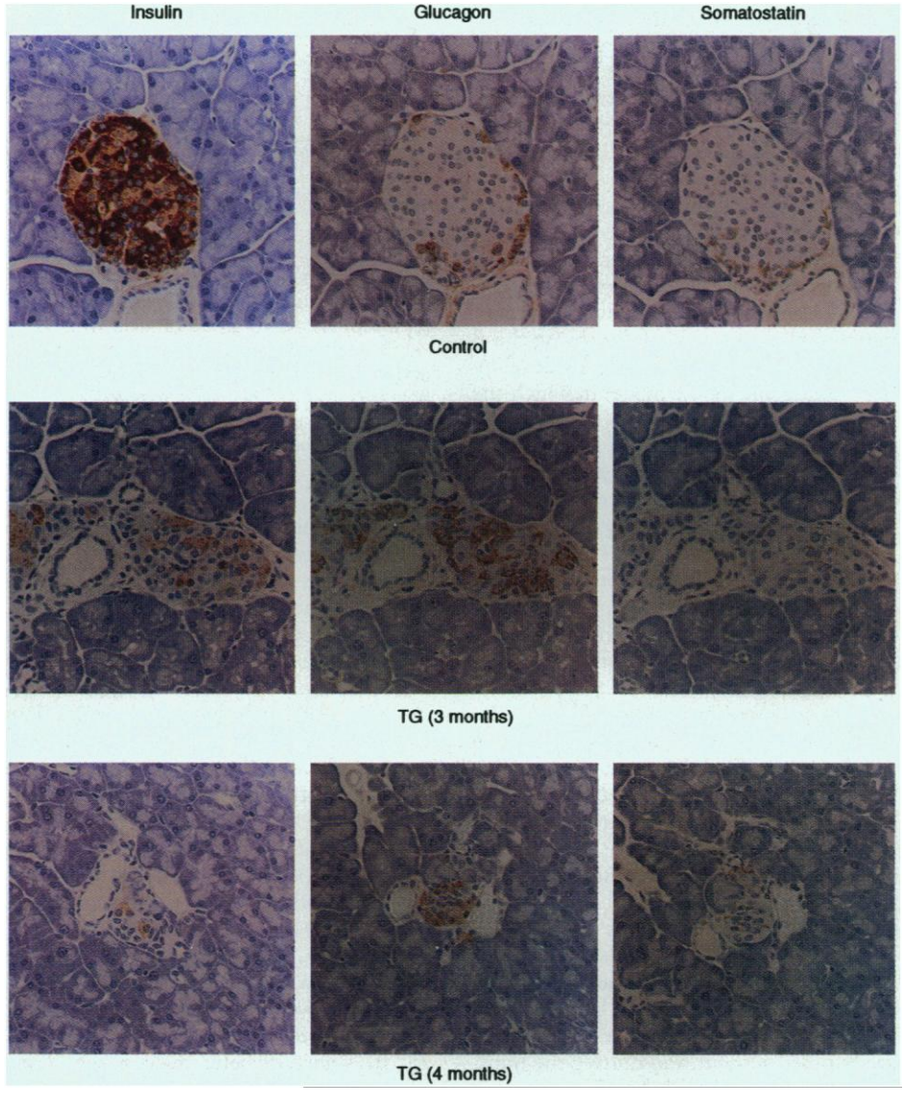

Fig. 3. Long-term diabetic mice have reduced absolute and relative numbers of insulin-containing cells and insulin staining. Coincident with the reduction in the number of insulin-containing cells, the relative number of glucagon- and somatostatin-containing cells appears to increase. We identified nontransgenic and transgenic mice by dot blot hybridization of DNA taken from tail biopsies and identified transgenic diabetic mice by venous blood analysis. The appropriate mice were killed by cervical dislocation and the pancreata fixed in 10% formalin. The tissues were embedded in paraffin, and then sections were cut and stained with biotinylated polyclonal antibodies against insulin, glucagon, or somatostatin. Using a commercially available kit (Vectastain), we developed a color reaction in the tissue sections stained with the antibodies, and the sections were then counterstained with hematoxylin. Shown is a comparison between a control mouse and diabetic transgenic (TG) mice at 3 and 4 months of age. Note the loss of insulin-staining cells and relative increase in glucagon- and somatostatin-containing cells that becomes more pronounced with increasing duration of diabetes. A comparable selective loss of β cells also occurs in human diabetics (*1*).

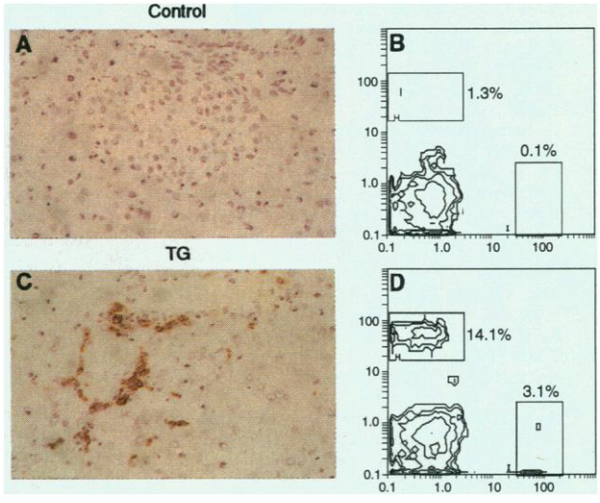

Fig. 4. The insulitis in the transgenic mice includes CD4- and CD8-positive lymphocytes. Pancreata from a control mouse (A and B) or transgenic mouse (C and D) were stained with a mAb against CD4 (**A** and **C**) or were used to generate a single cell suspension that was stained with antibodies against CD4 and CD8 (**B** and **D**). We prepared the pancreata by either removing and snap-freezing them in optimal cutting temperature (OCT) (Tissue-Tek) compound for sectioning or removing the pancreata after whole-body perfusion with PBS for fluorescence-activated cell sorting (FACS) analysis. Pancreatic sections were stained with a biotinylated antibody against CD4, color-developed with a Vectastain kit, and counterstained with eosin. Perfused pancreata were sequentially digested with collagenase and deoxyribonuclease. The first digestion was for 12 min [collagenase (5 mg/ml)], the second digestion was for 5 min [collagenase (2.5 mg/ml)], and the third was for 5 min [collagenase (1.25 mg/ml)]. Each digestion mix contained the same amount of deoxyribonuclease (2.5 mg/ml). Large aggregates were removed by settling, and the single cell suspension was washed in a solution of PBS and 1% fetal calf serum. These cells were stained with phycoerythrin-conjugated antibody to CD4 (PharMingen, 01075A) and fluoroscein isothiocyanate–conjugated antibody to CD8 (Caltag, RM2201). We analyzed these stained and washed cells with an Elite flow cytometer. Initially, 50,000 non-gated events were collected; these were subsequently gated by side and forward scatter. Shown is a two-dimensional representation of the cells that have side and forward scatter characteristic of lymphocytes (determined from an analysis of splenocytes analyzed as part of the same experiment). The numbers in each plot represent the percentage of gated cells that stain with the two antibodies. The data shown are representative of the 10 control and 14 diabetic transgenic mice analyzed.

strating that β cells are unusually sensitive to NK- (*22*) and macrophage-mediated lysis (*23*). Furthermore, in response to IFN-α, peripheral monocytes and bone marrow cultures (non-T and non-B) will produce IL-1 (*24*), and activated T cells will produce IL-2 (*25*). Thus, once the process is initiated by local production of IFN-α, a powerful inflammatory reaction involving T and B cells, as well as NK cells and macrophages, could occur.

The production of IFN-γ by the β cells in transgenic mice also leads to a hypoinsulinemic diabetes (*13*). However, the diabetes induced in transgenic mice by the expression of IFN-α that we report here is more relevant to human diabetes for several reasons. First, IFN-α, rather than IFN-γ, is expressed by the β cells of type I diabetics. Second, in the islets of human diabetics, histologically obvious changes occur before the lymphocytes capable of making IFN-γ appear (*17*). Our recent development of mice lacking IFN-γ (*26*) will allow a genetic analysis of the importance of IFN-γ in the IFN-α diabetic mice, as well as in other mouse models of diabetes.

The induction of IFN-α by viruses is normally a transient phenomenon (*27*). However, this protein was detected in the islets of patients who had been clinically diabetic for many months (*17*). Viral induction of interferon gene transcription will also induce a factor termed IRF-1, and this factor can induce the transcription of reporter genes containing DNA sequences corresponding to IFN-α regulatory regions (*28*). These results suggest the possibility of an autostimulatory loop. However, the role of IRF-1 in the induction of the endogenous IFN-α genes is unclear (*29*). Alternatively, a chronic nonproductive viral infection may be responsible for maintaining IFN-α expression. Monocytes can produce IFN-α when these cells are cocultured with cell lines infected with, but not producing, either human immunodeficiency virus (*30*) or mumps virus (*31*). Because the majority of the islets in diabetic patients appear to synthesize interferon, it would be necessary to argue that most of the islets become chronically infected. A third possibility to explain the persistent interferon expression is that a positive feedback loop is initiated but that this loop involves other cells and factors in addition to the endocrine cells and IFN-α.

Table 1. We generated transgenic mice by mating diabetic males with either non-transgenic female siblings or CD1 female mice. Litters that were derived from the same male parent and that were born within 24 hours of each other were pooled. At 10 days of age the pups were randomly separated into two groups. All transgenic mice received mAb injections three times per week. For mice between the ages of 2 and 3 weeks, each injection consisted of 200 μg of antibody in 0.125 ml of PBS. Between 3 and 6 weeks, each injection consisted of 400 μg of antibody in 0.25 ml. All injections were subcutaneous. The monoclonal anti–IFN-α, LI-8, is a mouse immunoglobulin G1 (*33*) and had a neutralizing capacity of 300 U of consensus recombinant IFN-α per microgram of protein. The isotype-matched control was a mAb to tissue plasminogen activator from Chinese hamster ovary. From 3 to 6 weeks sera glucose concentrations were determined from tail venous blood with a Lifescan glucose analyzer. The presence of diabetes was defined as two or more consecutive glucose readings of greater than 200 mg/dl. At 6 weeks the transgenic mice were killed, and the pancreata were removed and fixed in formalin. For technical reasons, not all pancreata were available for histological analysis, and in some sections islets were not present, which prevented us from evaluating insulin staining. We analyzed paraffin-embedded hematoxylin- and eosin-stained sections for the extent of insulitis and insulin staining. We evaluated the histological sections without knowing which group of mice they came from and scored them for severity of insulitis and intensity of insulin staining. The intensity of insulin staining in an untreated nontransgenic mouse would be assigned a score of between 5 and 6.

Treatment group	Diabetes incidence at week (affected/total)				Severity of insulitis (number of mice)						Insulin staining (score)						
	3	4	5	6	0*	1	2	3	4	5	0†	1	2	3	4	5	6
Control	8/61	13/61	15/61	18/61‡	0	4	7	25	17	1	1	4	18	17	7	6	1
Anti–IFN-α	3/50	3/50	3/50	4/50‡	11	14	14	6	2	0	0	1	0	2	4	22	17

*Severity of insulitis was scored on a scale of 0, minimal, to 5, severe. The mean score for mice treated with the control mAb was 3.2 and with the anti–IFN-α, 1.5. ($P < 0.001$ by Cochran-Mantel-Haenszel test.) †Intensity of insulin staining was scored on a scale of 0, minimal, to 6, very intense. The mean score for control mice was 3.1 and for anti–IFN-α mice, 5.1. ($P < 0.001$ by Cochran-Mantel-Haenszel test.) ‡$P < 0.01$ by chi-square test.

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16. In addition to lesions within the islets of transgenic mice, the exocrine pancreas had changes that varied considerably among animals. Changes included tracts of acinar necrosis, atrophy, or both with accompanying infiltration of mixed populations of mononuclear inflammatory cells into the interstitium. Typically associated with affected portions of exocrine pancreas were ductile-like structures with intimately associated islets. Inflammation also involved these ductiles and associated islets.
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Nitric Oxide and Carbon Monoxide Produce Activity-Dependent Long-Term Synaptic Enhancement in Hippocampus

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Nitric oxide (NO) and carbon monoxide (CO) may act as retrograde messages for long-term potentiation (LTP) in the hippocampus. Zinc protoporphyrin IX, an inhibitor of the enzyme that produces CO, blocked induction of LTP in the CA1 region of hippocampal slices. Application of either NO or CO to slices produced a rapid and long-lasting increase in the size of evoked synaptic potentials if, and only if, the application occurred at the same time as weak tetanic stimulation. This long-term enhancement was spatially restricted to synapses from active presynaptic fibers and appeared to involve mechanisms utilized by LTP, occluding the subsequent induction of LTP by strong tetanic stimulation. The enhancement by NO and CO was not blocked by an *N*-methyl-D-aspartate (NMDA) receptor blocker, suggesting that NO and CO act downstream from the NMDA receptor. Also, CO produced long-term enhancement when paired with low-frequency stimulation. These results are consistent with the hypothesis that NO and CO, either alone or in combination, serve as retrograde messages that produce activity-dependent presynaptic enhancement during LTP.

Long-term potentiation is a type of synaptic plasticity that is thought to contribute to certain forms of learning in mammals (*1*). Whereas induction of LTP in the CA1 region of the hippocampus requires Ca^{2+} influx through postsynaptic NMDA receptor channels, maintenance of LTP apparently involves in part a presynaptic increase in transmitter release, implying that the postsynaptic cell must send one or more retrograde messages to the presynaptic terminals (*2–5*). Previous experiments with inhibitors of NO synthase have indicated that the membrane-permeant molecule NO may be such a message (*2–4*). Inhibitors of NO synthase also impair certain kinds of learning in vivo (*6*). To determine how NO contributes to LTP, we examined the effects of applying NO directly to hippocampal slices. In addition, we compared these actions with those produced by CO because it has actions similar to those of NO in other systems (*7, 8*).

A diffusible retrograde message could spread laterally and potentiate transmission at inactive presynaptic terminals, in contrast with the observed pathway specificity of LTP (*9*). A possible solution would be for the message to be effective only at recently active presynaptic fibers, as during activity-dependent presynaptic facilitation in *Aplysia* (*10*). We tested this possibility by applying NO to hippocampal slices either alone or paired with weak tetanic stimulation of the presynaptic fibers that by itself produced little or no LTP (*11*). Alone, NO (0.1 to

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