growth of V-K1735 for 90 days, whereas tumors appeared in 80% of naïve mice by day 18 (Fig. 4). Thus, B7-K1735 can effectively prime for a response against challenge by the control V-K1735, which demonstrates that B7-K1735 and V-K1735 are immunologically cross-reactive. This supports the notion that the costimulatory activity provided by the B7 gene product is the functionally important difference between these tumors.

In order to directly test whether $CD4^+$ T cells, $CD8^+$ T cells, or both subsets were responsible for the rejection of B7-K1735, mice were depleted of $CD4^+$ cells, $CD8^+$ cells, or both, by antibody treatments in vivo before implanting B7-K1735. As expected, B7-K1735 tumors grew aggressively in mice depleted of both $CD4^+$ and $CD8^+$ T cells, demonstrating that T cells are responsible for rejection of B7-K1735 in intact mice (Fig. 5A). Growth of B7-K1735 was greatly enhanced in CD8-depleted mice relative to nondepleted mice, demonstrating that $CD8^+$ cells are required for the rejection of B7-K1735 (Fig. 5B). In contrast, tumor growth was only slightly enhanced in CD4-depleted mice, demonstrating that $CD4^+$ cells are not necessary for rejection (Fig. 5C). These results demonstrate that "help" from $CD4^+$ cells does not seem to be necessary for the induction of $CD8^+$ activity in this system, and that $CD8^+$ cells are primarily responsible for the rejection of B7-K1735.

Exogenous help by $CD4^+$ T cells can augment a cytotoxic T lymphocyte (CTL) response (*24–27*). However, effector $CD8^+$ CTL can be generated in the absence of $CD4^+$ cells, provided that accessory cells are present (*28–31*). We have found that costimulation provided by the B7-CD28 interaction allows the generation of allogeneic CTL from naïve precursors in the absence of $CD4^+$ cells, and that costimulation is not required for effector function (*12*). The results presented here are consistent with these observations. The lack of growth of the B7-K1735 tumor in CD4-depleted mice and the growth in CD8-depleted mice suggests that B7 can directly costimulate $CD8^+$ T cells in vivo, independent of the presence of $CD4^+$ T cells. In addition, the protection conferred by the B7-K1735 tumor to subsequent challenge by V-K1735 suggests that the expression of B7 is not necessary for killing in vivo.

We have shown that provision of costimulation by surface expression of B7 provides protection against the growth of a melanoma in vivo. This protection is mediated primarily by $CD8^+$ T cells, which can function in the absence of help from $CD4^+$ cells. Other studies have focused on boosting anti-tumor immune responses by providing "help" in the form of lymphokines normally produced by $CD4^+$ T cells (*32–34*). Manipulation of costimulation offers another strategy for tumor immunotherapy. Direct activation of CTL by tumors with appropriate costimulation may "jump-start" the anti-tumor response by bypassing the need for exogenous help, which could lead to a faster, more effective anti-tumor response in situ. This may provide a means to elicit effective CTL responses to established tumors in the immunotherapy of primary or metastatic disease.

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Cyclic ADP–Ribose in Insulin Secretion from Pancreatic β Cells

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Inositol 1,4,5-trisphosphate (IP_3) is thought to be a second messenger for intracellular calcium mobilization. However, in a cell-free system of islet microsomes, cyclic adenosine diphosphate–ribose (cADP-ribose), a nicotinamide adenine dinucleotide (NAD^+) metabolite, but not IP_3, induced calcium release. In digitonin-permeabilized islets, cADP-ribose and calcium, but not IP_3, induced insulin secretion. Islet microsomes released calcium when combined with the extract from intact islets that had been incubated with high concentrations of glucose. Sequential additions of cADP-ribose inhibited the calcium release response to extracts from islets treated with high concentrations of glucose. Conversely, repeated additions of the islet extract inhibited the calcium release response to a subsequent addition of cADP-ribose. These results suggest that cADP-ribose is a mediator of calcium release from islet microsomes and may be generated in islets by glucose stimulation, serving as a second messenger for calcium mobilization in the endoplasmic reticulum.

Glucose is the primary stimulus of insulin secretion and synthesis from the pancreatic islets of Langerhans (*1*, *2*). Increases in intracellular Ca^{2+} concentration mediate the biochemical events that couple glucose stimulation to insulin secretion by the islets, and mobilization of Ca^{2+} from intracellular stores in the endoplasmic reticulum is important in this process (*3*). It has been thought that IP_3 is a second messenger for Ca^{2+} mobilization from intracellular stores (*4*). However, some aspects of agonist ac-

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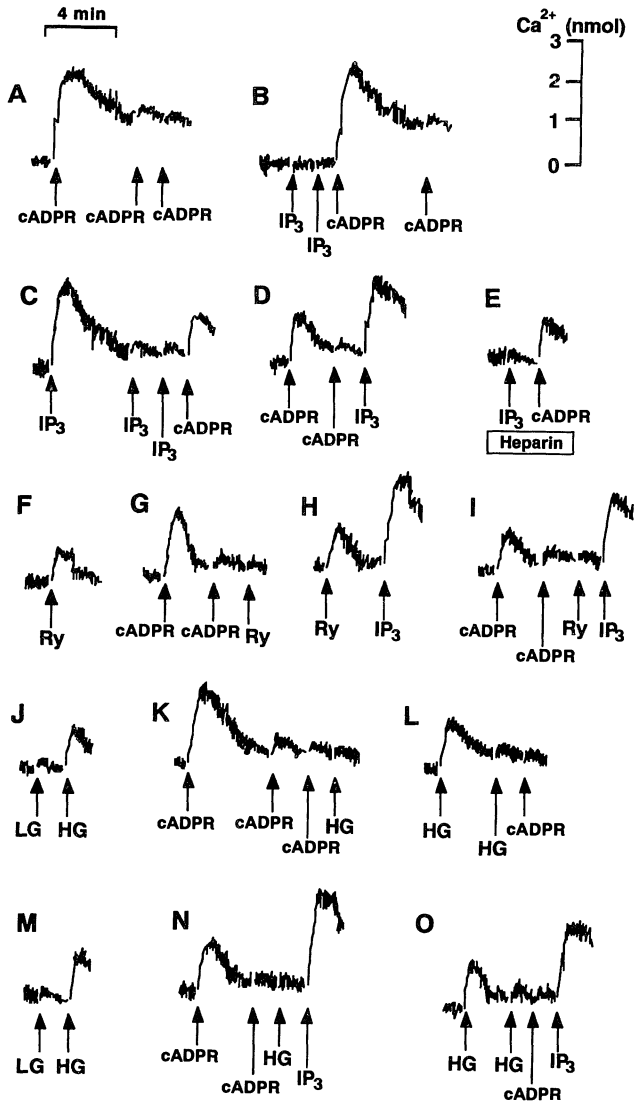

Fig. 1. Release of Ca^{2+} induced by cADP-ribose and IP_3 from islet (**A**, **B**, **F**, **G**, and **J** through **L**) and cerebellum (**C** through **E**, **H**, **I**, and **M** through **O**) microsomes measured fluorometrically with fluo 3. Cyclic ADP-ribose (cADPR) (1.0 μM), IP_3 (1.0 μM), heparin (100 μg/ml), ryanodine (Ry) (100 μM), and a 10-μl aliquot of the high- or low-glucose (HG or LG)–treated islet extract were added where indicated. Fluo 3 fluorescence was measured at 490-nm excitation and 535-nm emission. The absolute amount of Ca^{2+} released is indicated on the ordinate. Breaks in the record occurred during additions to the cuvette.

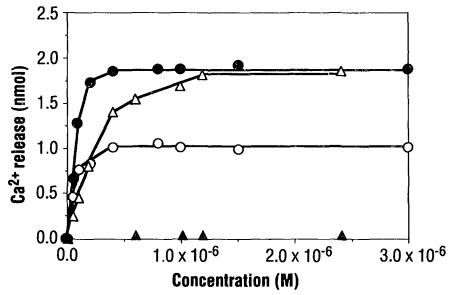

Fig. 2. Concentration response of cADP-ribose and IP_3 to release Ca^{2+} from islet and cerebellum microsomes; Ca^{2+} release from islet microsomes by cADP-ribose (●) and by IP_3 (▲), and from cerebellum microsomes by cADP-ribose (○) and by IP_3 (△). The release of Ca^{2+} was measured as described in the legend to Fig. 1. The absolute amount of Ca^{2+} release is indicated on the ordinate.

tion cannot be accounted for solely on the basis of an IP_3 mechanism (5). In freshly obtained human insulinoma cells, glucose induces insulin secretion without altering inositolphospholipid turnover (6). Recently, cADP-ribose, a metabolite of NAD^+, has been reported to be as potent and powerful a releaser of intracellular Ca^{2+} as IP_3 in sea urchin eggs and permeabilized rat pituitary cells (7, 8). We have now investigated the role of IP_3 and cADP-ribose in insulin secretion from pancreatic β cells.

We used rat islet microsomes as a cell-free system to study Ca^{2+} release (9, 10) and found that cADP-ribose (11) induced Ca^{2+} release from islet microsomes as indicated by a prompt increase in 1-[2-amino-5-(2″,7″-dichloro-6″-hydroxy-3″-oxy-9″xanthenyl)-phenoxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (fluo 3) fluorescence (Fig. 1); repeated additions of cADP-ribose attenuated the Ca^{2+} release response to cADP-ribose. The release of Ca^{2+} after additions of cADP-ribose showed a steep dose-response relation with a clear effect at concentrations as low as 0.09 μM and a near maximum release at 0.5 μM (Fig. 2). However, although the addition of IP_3 alone did not cause a release of Ca^{2+} from islet microsomes, the subsequent addition of cADP-ribose did release Ca^{2+} from the microsomes (Fig. 1B). In contrast, IP_3 induced Ca^{2+} release from rat cerebellum microsomes, and repeated additions of IP_3 attenuated the Ca^{2+} release response to IP_3 (Fig. 1C). After these additions, the microsomes were also responsive to additions of cADP-ribose. Repeated additions of cADP-ribose attenuated the Ca^{2+} release response to cADP-ribose (Fig. 1D). After these additions, the microsomes were still responsive to additions of IP_3. The dose-response curves of cADP-ribose and IP_3 on Ca^{2+} efflux from cerebellum microsomes were dose-dependent, with half-maximal release occurring at 0.09 μM cADP-ribose and 0.15 μM IP_3, and maximal Ca^{2+} release occurring at 0.8 μM cADP-ribose and 1.2 μM IP_3 (Fig. 2). These results indicate that, in contrast to islet microsomes, cerebellum microsomes respond to both cADP-ribose and IP_3, but cADP-ribose appears to induce a Ca^{2+} release from cerebellum microsomes by a mechanism different from that utilized by IP_3.

Moreover, heparin, an inhibitor of IP_3 binding to its receptor (12), blocked IP_3-induced Ca^{2+} release from cerebellum microsomes but did not block cADP-ribose–induced Ca^{2+} release (Fig. 1E). Heparin did not affect the response to cADP-ribose in islet microsomes (13). Ryanodine also induced a Ca^{2+} release from islet and cerebellum microsomes (Fig. 1, F and H); microsomes treated with cADP-ribose were desensitized not only to itself but also to ryanodine (Fig. 1, G and I), although IP_3 could still trigger a large Ca^{2+} release from cerebellum microsomes (Fig. 1I). These observations are consistent with results observed in sea urchin eggs (14), where the important link between cADP-ribose and Ca^{2+}-induced Ca^{2+} release was first demonstrated. Islam and co-workers reported a Ca^{2+}-induced Ca^{2+} release in insulin-secreting cells (15). Cyclic ADP–ribose may participate in this Ca^{2+} release as an intracellular signal molecule.

We then examined the effects of cADP-ribose and IP_3 on insulin secretion by using digitonin-permeabilized pancreatic islets (16). Both cADP-ribose (1 μM) and Ca^{2+} (0.2 μM) induced insulin secretion, but IP_3 did not (Fig. 3). The combined addition of cADP-ribose and Ca^{2+} did not induce significantly more insulin secretion than the addition of cADP-ribose or Ca^{2+} alone. The cADP-ribose–induced insulin secretion was inhibited by the addition of EGTA. Dose-response relations between cADP-ribose and insulin secretion from permeabilized islets are shown in Fig. 4. Near maximal secretion of insulin by cADP-ribose was observed at 0.5 μM and half-maximal secretion at 0.09

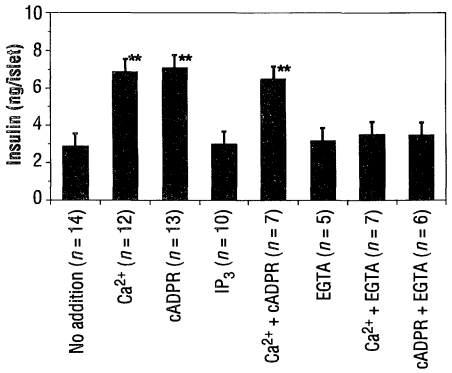

Fig. 3. Effect of cADP-ribose on insulin secretion from digitonin-permeabilized islets. Permeabilized islets were treated with cADP-ribose (1 μM), IP_3 (1 μM), Ca^{2+} (0.2 μM), and cADP-ribose (1 μM) in the presence of Ca^{2+} (0.2 μM), and with cADP-ribose (1 μM) in the presence of EGTA (10 mM). Values represent means ± SEM of $n = 5$ to 14 measurements. Results of two-tailed Student's t test comparing the response to each addition versus the control (no addition) are shown. $^{**}P < 0.01$.

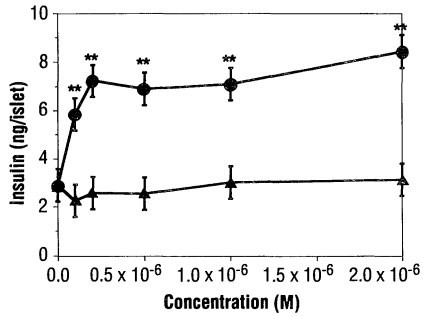

Fig. 4. Dose response of cADP-ribose and IP_3 to release insulin from digitonin-permeabilized pancreatic islets; insulin release by cADP-ribose (●) and by IP_3 (▲). The experiments were performed four times (cADP-ribose) or three times (IP_3), and the results were reproducible. The SEM is indicated. Results of two-tailed Student's t test comparing response to each addition as compared to the control (no addition) are shown. $^{**}P < 0.01$.

Table 1. Effects of streptozotocin, nicotinamide, and 3-aminobenzamide on the Ca^{2+} mobilizing activity in islet extracts. Values are mean ± SEM.

Addition (n)	Ca^{2+} mobilizing activity (nmol per 100 islets)
2.8 mM glucose (6)	0.23 ± 0.04
20 mM glucose (10)	0.79 ± 0.04*†
20 mM glucose and 2 mM streptozotocin (6)	0.18 ± 0.03
20 mM glucose, 3 mM nicotinamide, and 2 mM streptozotocin (3)	0.65 ± 0.08*†
20 mM glucose, 0.4 mM 3-aminobenzamide, and 2 mM streptozotocin (3)	0.73 ± 0.07*†
20 mM glucose and 3 mM nicotinamide (2)	0.88 ± 0.08*†
20 mM glucose and 0.4 mM 3-aminobenzamide (2)	0.74 ± 0.09†

*$P < 0.01$ when compared to 2.8 mM glucose. †$P <$ 0.01 when compared to 20 mM glucose and 2 mM streptozotocin. The Ca^{2+} mobilizing activity of islet extracts was measured with cerebellar microsomes (*9*).

μM. The dose-response curve was well fitted to that of Ca^{2+} release from islet microsomes (Fig. 2). These results suggest that the cADP-ribose–induced insulin secretion was mediated by Ca^{2+} mobilization from islet microsomes.

When the extract from intact islets incubated with 2.8 mM or 20 mM glucose (*17*) was added to a cell-free system of islet microsomes, a prominent Ca^{2+} release was found with the extract from islets treated with high concentrations of glucose (HG), but not with extracts from islets treated with low concentrations of glucose (LG) (Fig. 1J). Repeated additions of cADP-ribose attenuated the Ca^{2+} release response to cADP-ribose, which may suggest a depletion of a cADP-ribose–responsive Ca^{2+} pool in the microsome. Subsequently, the microsomes were also no longer responsive to an addition of the HG-treated islet extract (Fig. 1K). After repeated additions of the islet extract, the microsomes were no longer responsive to additions of cADP-ribose (Fig. 1L). We incubated islets with 20 mM glucose for 3 to 40 min, prepared the islet extract, and measured its Ca^{2+} mobilizing activity. The Ca^{2+} mobilizing activity in the HG-treated islets attained near maximal level by 3 min (*13*). When the extract from islets incubated with glucose was added to cerebellar microsomes, Ca^{2+} release was seen with the HG-treated islet extract but not with the LG-treated islet extract (Fig. 1M). Repeated additions of cADP-ribose attenuated the Ca^{2+} release response to cADP-ribose. At this point, the cerebellar microsomes were also no longer responsive to additions of the HG-treated islet extract (Fig. 1N). After repeated additions of the islet extract, the cerebellar microsomes were also no longer responsive to additions of cADP-ribose (Fig. 1O). After these additions, the microsomes were still responsive to additions of IP_3 (Fig. 1, N and O). These results suggest that cADP-ribose, or a cADP-ribose–like molecule that targets the same Ca^{2+} pool as cADP-ribose, is formed during incubation of islets with HG.

Streptozotocin produces diabetes mellitus in experimental animal models. We have proposed that β-cytotoxins such as streptozotocin and alloxan cause DNA strand breaks that activate nuclear poly(ADP-ribose) synthetase, an enzyme that polymerizes the ADP-ribose moiety of NAD^+ to form poly(ADP-ribose), thereby depleting intracellular NAD^+ and inhibiting cellular functions in pancreatic islets (*18–20*). Poly(ADP-ribose) synthetase inhibitors such as nicotinamide and 3-aminobenzamide prevent the NAD^+ depletion through poly(ADP-ribose) (*18–20*). In fact, insulin secretion in islets is greatly reduced by streptozotocin and the reduction of insulin secretion is prevented by the poly(ADP-ribose) synthetase inhibitors (*21, 22*). We incubated rat pancreatic islets with streptozotocin, prepared the islet extract (*23*), and measured its Ca^{2+} mobilizing activity (Table 1). Streptozotocin greatly reduced the Ca^{2+} mobilizing activity in the islet extract, and nicotinamide (3 mM) and 3-aminobenzamide (0.4 mM) reversed this reduction. These results also suggest that an active component for Ca^{2+} release in the glucose-stimulated islet extract is cADP-ribose, as cADP-ribose is synthesized from NAD^+ (*8, 24–26*).

Our data indicate that cADP-ribose induces Ca^{2+} release from islet microsomes and causes insulin secretion in permeabilized islets. We have not yet been able to measure authentic cADP-ribose in LG and HG extracts because of the limited amount of tissue available (*26*). Although there is at present no way to exclude the involvement of molecules other than cADP-ribose in the islet extract, the fact that authentic cADP-ribose mimics the action of the glucose-treated islet extract together with the cross-desensitization results argue that the active component generated in the glucose-treated islet extract is either cADP-ribose itself or a molecule very similar to cADP-ribose.

The present results also indicate that both cADP-ribose and IP_3 were active in the mobilization of Ca^{2+} from cerebellum microsomes, but islet microsomes respond only to cADP-ribose. This suggests that responses to cADP-ribose and IP_3 vary according to tissue or cell type. Prentki *et al.* reported that IP_3 mobilizes Ca^{2+} from a microsome-enriched fraction taken from a rat insulinoma (*27*), from the nonmitochondrial pool of RINm5F cells (*28*), and from digitonin-permeabilized rat pancreatic islets (*29*). The signal transduction pathway in transformed β cells may have been changed as a result of their transformation, causing them to become responsive to IP_3. In permeabilized islets, IP_3 induced only transiently a very small amount of Ca^{2+} release but did not cause insulin secretion (*29*). In the same experimental system, cADP-ribose was able to induce insulin secretion (Fig. 3).

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9. The pancreases of male Wistar rats (240 to 280 g) that had been fed ad libitum were removed. Two thousand islets were isolated (*18*) and then homogenized with a Pellet mixer (Treff, Degersheim, Switzerland) in 200 μl of acetate intracellular medium (AcIM) (*10*) composed of 250 mM potassium acetate, 250 mM *N*-methyl-glucamine, 1 mM $MgCl_2$, and 20 mM Hepes supplemented with 0.5 mM adenosine triphosphate (ATP), 4 mM phosphocreatine, creatine phosphokinase (2 U/ml), 2.5 mM benzamidine, and 0.5 mM phenylmethylsulfonyl fluoride. After the homogenates had been centrifuged for 45 s (13,000*g*), microsomes were prepared by Percoll density gradient centrifugation [D. L. Clapper and H. C. Lee, *J. Biol. Chem.* **260**, 13947 (1985)]. For cerebellum microsomes, rat cerebellum was homogenized with a Dounce-type glass tissue homogenizer and microsomes were then prepared as described above. Release of Ca^{2+} was monitored in 3 ml of intracellular medium (GluIM) (*10*) composed of 250 mM potassium gluconate, 250 mM *N*-methyl-glucamine, 1 mM $MgCl_2$, 20 mM Hepes (pH 7.2) containing 1 mM ATP, 4 mM phosphocreatine, creatine phosphokinase (2 U/ml), 2.5 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, and 3 μM fluo 3, a fluorescent Ca^{2+} indicator [J. P. Y. Kao, A. T. Harootunian, R. Y. Tsien, *J. Biol. Chem.* **264**, 8179 (1989)], with the addition of 150 μl of islet microsome fraction (11 μg of protein) or cerebellum microsome fraction (88 μg of protein). Fluorescence was measured at 490-nm excitation and 535-nm emission (*7*) at 37°C with a circulating water bath.
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11. Cyclic ADP–ribose, prepared enzymatically from NAD^+ with ADP–ribosyl cyclase purified (*24*) from ovotestes of *Aplysia kurodai*, a species common around the Japanese coast, was purified by high-performance liquid chromatography (HPLC) (*25*) and characterized by ^1H nuclear magnetic resonance and fast atom bombardment mass spectrometry [H. C. Lee, T. F. Walseth, G. T. Bratt, R. N. Hayes, D. L. Clapper, *J. Biol. Chem.* **264**, 1608 (1989)]. Cyclic ADP–ribose, thus obtained, showed a single peak on HPLC and a peak at a mass-to-charge ratio (*m/z*) 540.2 corresponding to molecular ion of $(M-H)^-$ on negative ion fast atom bombardment mass spectrometry.
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16. Islets were permeabilized by digitonin treatment [J. R. Colca, B. A. Wolf, P. G. Comens, M. L. McDaniel, *Biochem. J.* **228**, 529 (1985)]. In brief, freshly isolated islets were incubated in a modified Hepes-Krebs buffer (25 mM Hepes, 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.1% bovine serum albumin, pH 6.8) at 37°C with digitonin (20 μg/ml) (Merck) for 20 min. Thirty islets were randomly selected, washed three times in tris buffer (50 mM tris, 100 mM KCl, 5 mM $MgCl_2$, 0.1% bovine serum albumin, pH 6.8), and incubated at 24°C for 15 min in 100 μl of tris-Pipes buffer (55 mM tris, 11 mM KCl, 5 mM $MgCl_2$, 1.1 mM EGTA, 1 mM ATP, 10 mM phosphocreatine, creatine kinase (10 units/ml), 0.1% bovine serum albumin, pH 7.5). Islets were treated with O_2:CO_2 (19·1). At the end of the incubation period, the medium was withdrawn and a radioimmunoassay kit (Novo Nordisk Biolabs, Baysvaerd, Denmark) was used to quantify the released insulin with rat insulin used as the standard.
17. For preparing the glucose-treated islet extract, 500 islets were isolated from the pancreases of male Wistar rats (240 to 280 g) that had fasted for 48 hours; these islets were incubated with 2.8 mM or 20 mM glucose in 100 μl of Krebs-Ringer's bicarbonate medium (*2, 18*) for 40 min at 37°C, washed three times with cold AcIM, and homogenized in 50 μl of AcIM. The homogenates were centrifuged at 4100*g* for 15 min in a Sorvall HB-4 rotor, and the supernatant was used as the glucose-treated islet extract. No changes in fluorescence were observed when islet extracts were added to a GluIM-fluo 3 system without microsomes.
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Blood-Brain Barrier Penetration and in Vivo Activity of an NGF Conjugate

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Nerve growth factor (NGF) is essential for the survival of both peripheral ganglion cells and central cholinergic neurons of the basal forebrain. The accelerated loss of central cholinergic neurons during Alzheimer's disease may be a determinant of dementia in these patients and may therefore suggest a therapeutic role for NGF. However, NGF does not significantly penetrate the blood-brain barrier, which makes its clinical utility dependent on invasive neurosurgical procedures. When conjugated to an antibody to the transferrin receptor, however, NGF crossed the blood-brain barrier after peripheral injection. This conjugated NGF increased the survival of both cholinergic and noncholinergic neurons of the medial septal nucleus that had been transplanted into the anterior chamber of the rat eye. This approach may prove useful for the treatment of Alzheimer's disease and other neurological disorders that are amenable to treatment by proteins that do not readily cross the blood-brain barrier.

The degeneration of specific neuronal cell types during age-related neurological disorders such as Alzheimer's disease presents opportunities for neurotrophic therapies. For example, the degeneration of certain cholinergic neurons in patients dying with Alzheimer's disease has been linked to their memory impairment (*1*). If the loss of cholinergic neurons were responsible at least in part for the clinical symptoms of Alzheimer's disease, a treatment that limits

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