7. The pneumococcal isolates were received from Dr. V. M. Howie, Huntsville, Ala. Aspirates taken to relieve pressure in the ears of ill children were streaked on blood agar plates: pneumococcal colonies were transferred to blood agar slants, and sent to Dr. Gerald Schiffman, Department of Microbiology, Downstate Medical Center. In Dr. Schiffman's laboratory, the slants were incubated for a few hours, and the pneumococci were transferred to Neopeptone beef-heart infusion broth containing 2 percent defibrinated rabbit blood. I received these cultures from Dr. Schiffman, stored them in the refrigerator, and transferred each culture to fresh blood broth the evening before I intended to test the strain. All pneumococcal type designations are according to the Danish system of nomenclature.
8. H. P. Bernheimer, in preparation.
9. I thank S. Scribani for all electron microscopy. Supported by NIH grant AI 05173 and career scientist award from the Irma T. Hirschl Trust.

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Complete Reversal of Experimental Diabetes Mellitus in Rats by a Single Fetal Pancreas

Abstract. *Complete reversal of streptozotocin-induced diabetes mellitus in adult rats will follow transplantation of a single fetal pancreas if the organ is first grown in a normal syngeneic carrier before transfer to the diabetic recipient. Careful control of the blood sugar of diabetic recipients may enhance the function of a single donor organ and thus improve histocompatibility matching.*

Under appropriate conditions transplanted fetal pancreases can completely reverse streptozotocin-induced diabetes in adult rats in addition to their having many other advantages as donor tissues (*1*). Initially, pancreases were removed from embryos at the optimal time of gestation (16 to 17½ days) and placed under the kidney capsule of syngeneic adult male rats previously made diabetic 3 to 4 days prior to the transplantation through streptozotocin injection. Recipients were treated with 4 units of NPH insulin (neutral protamine Hagedorn) injected daily for 4 days after transplantation and then with 3 units for an additional 4 days to partially control the diabetes during establishment of the fetal pancreas. Under these conditions, four or more pancreases were required to reverse completely the diabetic state. Transplantation of three fetal pancreases resulted in less than complete reversal and two transplants only alleviated the diabetic state significantly in 50 percent of the recipients.

In the present study, by evaluating the importance of blood sugar control following pancreas transplantation, we have found that a single fetal pancreas can completely reverse streptozotocin-induced diabetes mellitus. This was accomplished by culturing the fetal organ in vivo in a normal syngeneic carrier for a period of time before transplanting it into a diabetic host. The results suggest that control of the diabetic state may be very important for further growth and optimal development of function of the beta cells when the fetal pancreas is transplanted into diabetic recipients.

Pancreases were removed from embryos (16 to 17½ days old) of an inbred strain of Lewis rats (Microbiological Associates). One or two pancreases were immediately placed beneath the capsules of the right kidneys of normal syngeneic adults (primary transplants). At weekly intervals the kidneys, containing the undisturbed pancreases, were transplanted with vascular anastomosis into uninephrectomized syngeneic adult male rats (secondary transplant) made diabetic by intravenous injection of streptozotocin (62.5 to 65 mg per kilogram of body weight) 3 to 7 days previously. Urine volumes and glucose concentrations were measured daily, and plasma glucose concentrations were measured weekly, throughout the experiment beginning 24 hours after the injection of streptozotocin. Diabetes was of equal severity in control and all experimental groups. At 12 to 30 weeks after the secondary transplantation, the pancreases were removed from the kidney surface, and the recipients were monitored to assure that the observed reversal of diabetes was due to insulin secretion from the transplanted fetal pancreas rather than to spontaneous recovery of the host pancreas. The removed pancreatic tissues were immediately frozen (in acetone and Dry Ice) and stored at −20°C for determination of insulin content by radioimmunoassay according to the double antibody method.

Secondary transplantation of one or two fetal pancreases into adult diabetic rats did not significantly affect the diabetic state after 1 or 2 weeks in a normal carrier rat, whereas after 3 weeks in a normal carrier, the diabetic state was completely reversed (Fig. 1). Mean plasma glucose concentrations in ten diabetic rats of this group was 412 ± 3 mg/dl before transplantation (Fig. 2A). Daily urine volumes of these rats were 50 ± 6 ml prior to transplantation and decreased to normal levels of 11 ± 0.4 ml 4 weeks later. Glucose excretion in the urine fell from 1.20 ± 0.05 g/day to 0.08 ± 0.009 g/day over the same period. Additional evidence for complete reversal of the diabetic state was provided by the steady gain in body weight (2 g per day) in the experimental animals in contrast to the weight loss (2 g per day) in untreated diabetic controls. The glucose disappearance rate after intra-arterial injection of 0.5 mg of glucose per gram of body weight was 3.2 ± 0.5 percent per minute, a value not significantly different from normal rats of the same strain (2.9 ± 0.2 percent per minute). The mean concentration of immunoreactive insulin in the plasma of the rats with transplants before glucose injection was 50 ± 7 microunits per milliliter, a value barely elevated ($P < .05$) above normal (27 ± 2 microunits per milliliter). The mean increase in plasma insulin in response to glucose was 53 ± 11 microunits per milliliter, statistically not significantly different from the response in normal rats (39 ± 4 microunits per milliliter). Plasma glucose, urine volume, and glucose remained normal in this group of rats for more than 10 weeks until removal of the pancreas transplants. After removal of the transplants (Fig. 2A) the mean plasma glucose concentration reached 255 ± 16 mg/dl at 1 week and 390 ± 8 mg/dl at 2 weeks, when the mean urine volume reached 62 ± 4 ml/day and urine glucose 1.6 ± 0.6 g/day.

Secondary transplantation of fetal pancreases after an initial period of 4 or 5 weeks in a normal carrier rat was not only less effective in reversing diabetes but led to greater variability in the results (Fig. 1). Of eight diabetic rats receiving a 4-week-old secondary transplant of one fetal pancreas (Fig. 2B), only one manifested complete reversal of the diabetic state (plasma glucose 140 mg/dl) and the other seven were partially reversed (mean plasma glucose 277 ± 7 mg/dl from 3 to 5 weeks). At 6 weeks after secondary transplantation there was an abrupt spontaneous failure of response in all eight of these animals, the mean plasma glucose concentration reaching 414 ± 9 mg/dl compared to 413 ± 4 mg/dl before transplantation. The daily urine volume in the seven rats during the period of partial reversal of diabetes was 44 ± 7 ml/day, and with recurrence of severity at 6 weeks reached 137 ± 9 ml/day. Urine glucose content followed a similar pattern increasing from 1.1 ± 0.3 g/day during the period of partial reversal of diabetes to 11.0 ± 0.7 g/day follow-

ing failure of response. Among seven diabetic rats receiving secondary transplants with two fetal pancreases (Fig. 2C), five had a complete reversal of diabetes which was permanent in four of them but failed in one beginning at 7 weeks. Two of this group of seven rats had only a partial reversal of diabetes followed by failure at 6 weeks.

As described previously (*1*), pancreases from fetal rats aged 16 to 17½ days, when placed beneath the kidney capsule of diabetic recipients, become almost exclusively endocrine organs by establishment or growth of the endocrine elements with gradual atrophy of the exocrine tissues. Four or more fetal organs were necessary to completely reverse the diabetic state by this method.

The present findings suggest that the growth of endocrine elements in a fetal pancreas may occur in the renal subcapsular site of a normal rat. In support of this is the recent report that a maximum increase of beta cell mass (23-fold) was obtained at 21 days after placement of 18-day fetal pancreases beneath the kidney capsules of normal syngeneic rats (*2*). Our results clearly indicate that a normal environment enhances the growth or at least the function of fetal beta cells because, in the earlier transplantation procedure, in which only partial control of hyperglycemia was achieved by daily injections of insulin, four or more pancreases of the same fetal age were required for complete reversal of the diabetic state. Hyperglycemia or other effects of diabetes in the host may inhibit beta cell growth or function. Deleterious effects of a high glucose content in the medium of culture rat islets supports this conclusion (*3*).

A hyperglycemic stimulus provided by secondary transplantation from the normal carrier into a diabetic recipient at the appropriate time appears to result in optimal development and maturation of fetal pancreas function. Transfer at approximately 3 weeks was optimal to reverse diabetes, but a delay to 4 weeks resulted in total long-term reversal of diabetes in only four of seven recipients.

Measurement of the immunoreactive insulin content of the transplants provides additional evidence for continued increase in insulin synthetic capacity by the islet tissue transplanted secondarily into diabetic recipients. Transplants removed from eight normal carriers after 3 to 4 weeks of growth contained 20 ± 4 milliunits of insulin per pancreas. In contrast, six pancreases secondarily transplanted into diabetic recipients and removed after several months of complete reversal of the diabetic state contained 108 ± 16 milliunits per pancreas. These results may be analogous to the finding that in adult rats after 90 percent of the pancreas is removed, hyperglycemia caused by either twice daily injections of glucose or injections of antiserum to insulin during the first 10 days, increases the number of islets and prevents late development of diabetes (*4*). For the fetal beta cells to be sufficiently mature to give a maximum response to hyperglycemia several weeks may be required. Fetal beta cells do not release insulin in response to glucose until a few days after birth, despite a high insulin content and despite their being responsive to phosphodiesterase inhibitors, such as caffeine and theophylline (*5*). Even islets removed from normal rats at 6 days after birth do not release as much insulin in vitro in response to a glycemic stimulus as do those obtained from adult rats, despite their having an equivalent insulin content (*6*).

An alternative explanation for the more favorable response to one fetal pancreas secondarily transplanted with the carrier kidney, compared with the primary transplantation, is the immediate vascular supply to the pancreas provided by the vascular anastomosis. This explanation seems unlikely because we observed good vascularization of the fetal organs within 24 to 48 hours after they were placed under the kidney capsule of the diabetic rat. That vascularization is inhibited by diabetes is also unlikely, because the rats were diabetic for only 3 to 4 days before transplantation and insulin injections were given for 8 days after the primary transplantation to modulate the severity of the diabetic state.

Abrupt and complete failure of diabet-

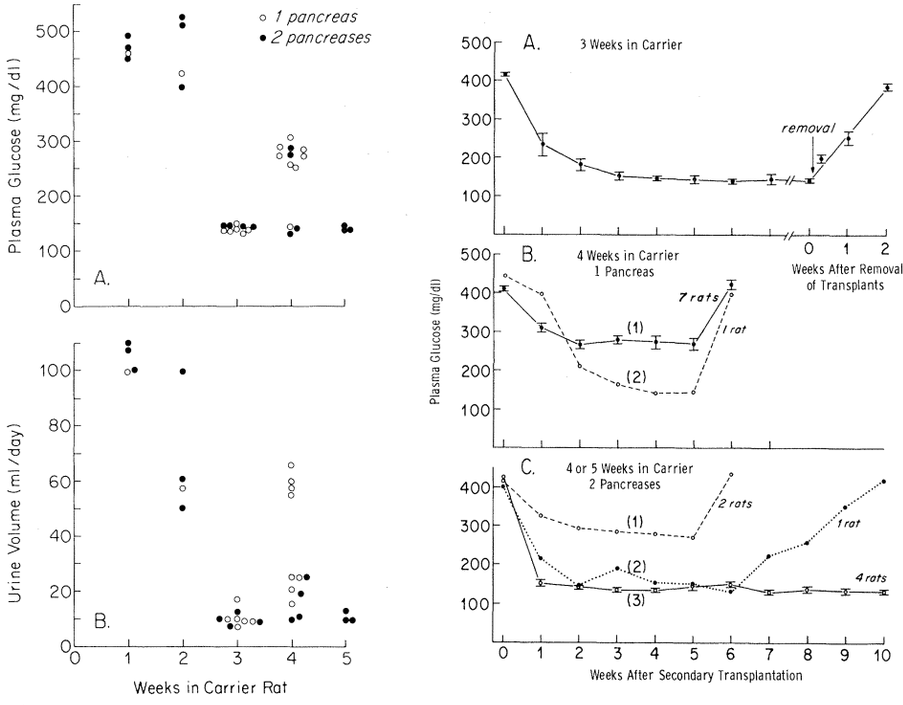

Fig. 1 (left). Plasma glucose (A) and urine volume (B) after secondary transplantation of fetal pancreases into diabetic rats after the pancreases had been various lengths of time in the carrier rat. The average plasma glucose concentration and urine volume for each diabetic rat during the 3 to 5 weeks after the secondary transplantation of one pancreas (○) or two pancreases (●) are plotted against the number of weeks of growth in a normal carrier rat before the secondary transplantation. Fig. 2 (right). Plasma glucose concentrations (mean ± standard error) of groups of diabetic rats measured before and at weekly intervals after the secondary transplantation of syngeneic fetal pancreases. (A) With ten rats being used as the mean, after 3 weeks growth in a normal carrier the transplantation of one pancreas (six rats) or two pancreases (four rats) into diabetic recipients resulted in a fall of plasma glucose to normal. After 10 or more weeks, the transplants were removed from the kidney surface; the mean glucose levels before and during the subsequent 2 weeks are shown. (B) After 4 weeks growth in a normal carrier, the transplantation of one pancreas resulted in: (1) partial temporary reversal of diabetes in seven rats and (2) complete reversal in one rat. At 6 weeks after transplantation, the plasma glucose concentration of all recipients spontaneously returned to the levels before transplantation. (C) After 4 or 5 weeks of growth in a normal carrier, the transplantation of two pancreases resulted in: (1) two rats exhibiting a partial temporary response followed by spontaneous recurrence of diabetes at 6 weeks, (2) one rat exhibiting complete reversal of diabetes but gradually showing a recurrence beginning at 7 weeks, (3) four rats achieving total reversal of diabetes of long duration.

ic control, when it occurred, usually did so at 6 weeks after the secondary transplantation almost exclusively in animals which had exhibited only partial reversal of the diabetic state. The immunoreactive insulin content of transplants removed from six animals displaying failure after a partial (five rats) or complete (one rat) reversal of diabetes was only 9 ± 3.3 milliunits per pancreas compared with 108 ± 16 milliunits per pancreas in rats with complete and permanent reversal of the diabetic state. The low insulin content of the transplants removed from rats following failure of control of diabetes may be due, in part, to intense stimulation of insulin secretion leading to depletion of stored insulin or, alternatively, failure of the pancreatic tissue to grow. To differentiate between depleted storage and limited insulin synthesis and release we would have to measure the secretory capacity of the transplants.

These results suggest the overstimulation of insulin secretion by hyperglycemia at an early stage after fetal pancreas transplantation may limit optimal growth and maturation of fetal beta cells and thus limit insulin secretion. To reduce the amount of pancreatic tissue needed to control the diabetic state, careful control of the blood sugar may be necessary. Since a single donor is desirable in allografts for proper histocompatibility matching to minimize problems of rejection, our observations may be of importance when this method is applied to human beings with diabetes mellitus.

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References and Notes

1. J. Brown, I. G. Molnar, W. Clark, Y. Mullen, *Science* **184**, 1377 (1974); J. Brown, W. R. Clark, I. G. Molnar, Y. S. Mullen, *Diabetes* **25**, 56 (1976); P. Mazur, J. A. Kemp, R. H. Miller, *Proc. Natl. Acad. Sci. U.S.A.* **73**, No. 11 (1976).
2. O. D. Hegre, R. J. Leonard, J. D. Rusin, A. Lazarow, *Anat. Rec.* **185**, 209 (1976).
3. R. Pictet and W. Imagawa, *Diabetes* **25** (Suppl. 1), 381 (1976).
4. J. M. Martin, W. H. Gregor, P. M. Lacy, R. G. Evens, *ibid.* **12**, 538 (1963).
5. A. E. Lambert, B. Jeanrenaud, A. E. Renold, *Lancet* **1967-I**, 819 (1967); K. Aspland, S. Westman, G. Hellerstrom, *Diabetologia* **5**, 260 (1969); M. Espinosa de los Monteros, A. S. G. Driscoll, A. J. Steinke, *Science* **168**, 1111 (1970); R. D. G. Milner, M. A. Ashworth, A. J. Barson, *Horm. Metab. Res.* **3**, 353 (1971); M. De Gasparo, R. L. Pictet, L. B. Rall, W. J. Rutter, *Develop. Biol.* **47**, 106 (1975).
6. V. Grill, K. Asplund, G. Hellerstrom, P. Cerasi, *Diabetes* **24**, 746 (1975).
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Salivary Gland Hyperglycemic Factor: An Extrapancreatic Source of Glucagon-Like Material

Abstract. *Extracts of homogenates of rat, mouse, rabbit, and human submaxillary salivary glands contain a significant quantity of a material with glucagon-like immunoreactivity. Fractionation of this material on columns of Sephadex G-100 reveals a single peak immediately following a gamma globulin marker but in advance of a rat growth hormone marker, crystalline amylase, and isotopically labeled porcine insulin and glucagon. This material, which is urea stable, shows identical immunoassay dilution curves when measured with the highly specific K-30 glucagon antiserum. Study of paired glands in vitro shows that low concentrations of glucose stimulate and high concentrations of glucose suppress release of this material. Arginine promotes brisk release in vitro. Somatostatin does not influence arginine-stimulated secretion and insignificantly suppresses basal release in vitro. These findings lend support to previous speculations that the salivary glands may possess endocrine as well as exocrine functions. Salivary gland glucagon may also be the source of circulating glucagon recently reported in pancreatectomized and eviscerated rats.*

By means of the glucagon radioimmunoassay, it was demonstrated that, as with other peptide hormones (*1*), there is considerable molecular heterogeneity among, as well as several sources of, circulating immunoreactive glucagons. Further, it was shown that "pancreatic-specific" antiserums raised to pancreatic glucagon could cross-react with some of these non-islet glucagons. A plasma glucagon of serum globulin dimensions has been observed (*2*), and several laboratories have investigated immunoreactive glucagon-like materials of enteric origin (*3*). There are significant differences in the elution chromatography profiles of serum glucagons in different populations of normal and diseased humans (*4*). In pancreatectomized dogs, glucagon concentrations, measured with a widely used pancreatic-specific glucagon antiserum (*5*), persist and even rise (*6, 7*). Furthermore, arginine, which is known to stimulate the secretion of pancreatic glucagon, elicits a brisk increase in the plasma glucagon concentration in dogs several days after they have been pancreatectomized (*7*).

It was also reported that true pancreatic alpha cells were present in the gastric fundus of dogs and that extracted glucagon from this source possessed essentially all of the physicochemical characteristics of pancreatic glucagon (*8*). A plausible gastrointestinal source of pancreatic glucagon was thus offered to explain the increasing concentrations of glucagon in pancreatectomized animals. However, soon thereafter, it was reported that glucagon levels almost triple over a 48-hour period in eviscerated and pancreatectomized rats maintained alive with a functional liver (*9*). It was these discoveries that prompted us to look for additional extrapancreatic sources of glucagon.

We have found a significant amount of a large immunoreactive glucagon-like material in the submaxillary salivary glands of rabbits, rats, and mice, and a measurable amount in the submaxillary glands of humans (Table 1). Insignificant amounts are present in the parotid and sublingual glands of rodents and man. Small amounts of a glucagon-like immunoreactive substance were reported earlier in the submaxillary glands (*10*), but using the cited method of extracting these tissues, we were unable to confirm such findings. The apparent discrepancy stemmed from the fact that immunoreactive glucagon of salivary gland origin is a large peptide that is not extractable by the traditional acid-alcohol method of Kenny (*11*) used in that study. The peptide can be obtained, however, by simple, acid-saline extraction in the presence of Trasylol, an effective protease inhibitor. After thorough homogenization in 0.9 percent saline acidified with 0.1*N* HCl to *p*H 2.8, samples from the submaxillary glands of the rat are spun at 2000 rev/min for 15 minutes, and the supernatant is removed and frozen. It is then thawed and recentrifuged, and the supernatant is applied to a Sephadex column (G-100; 50 by 1.5 cm) and eluted with either 0.2*M* glycine buffer, *p*H 7.5, or with 0.09 percent NaCl.

Using this approach we find significant amounts of a large immunoreactive glucagon-like material which appears in a discrete peak of fractions immediately following an isotopically labeled gamma globulin marker, suggesting a molecular weight of 70,000. This peak retains its chromatographic profile after repeated chromatography and after incubation with 8*M* urea and 1*M* acetic acid for 16 hours. Serial dilutions of this glucagon-like material show identical immunoassay curves when measured with the Unger K-30 glucagon antiserum, even though the column elution pattern for