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Studies on Rats with Islet Beta Cell Tumors Induced by Nicotinamide and Streptozotocin (39368)

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Rakieten *et al.* (1) first reported that the combined administration of streptozotocin and nicotinamide resulted in the induction of pancreatic islet tumors containing high concentrations of immunoreactive insulin (IRI). It was felt that this rat islet adenoma may provide a suitable experimental model for human insulinoma. This report details observations on the physiologic responses of rats following tumor induction, and on the insulin biosynthetic activity of the tumors. A portion of this work has appeared in abstract form (2).

Methods and materials. Young Holtzman rats weighing 180 g were fasted overnight and injected ip with nicotinamide (350 mg/kg body weight in 0.85% saline). Ten minutes later a single iv injection of streptozotocin (65 mg/kg body weight in a citrate buffer, pH 4.5) was administered, followed by another ip dose of nicotinamide 180 min after the first dose, as described by Rakieten *et al.* (1). The control rats were treated with nicotinamide and citrate buffer. All the animals were weighed weekly and bled monthly by tail vein. Blood glucose was determined with an AutoAnalyzer by a modification of the method of Hoffman (3).

Glucose tolerance test (GTT). The animals were fasted overnight and 30% glucose solution in 0.85% saline was administered by tail vein (1 ml per 100 g body weight). Blood sugar values were determined on samples withdrawn before and ½, 1 and 2 hr after glucose injection. The Diabetic Index (DI), which determined the ability of the animals to handle sudden glucose influx, was calculated by the following equation of Coupland *et al.* (4):

$$DI = \frac{\text{blood sugar at 1 hr} \times \text{blood sugar at 2 hr of experimental animals}}{\text{blood sugar at 1 hr} \times \text{blood sugar at 2 hr of control animals}}$$

The animals were anesthetized with Pentosol (Sodium pentabarbital 50 mg/ml, Invenex, California), and bled by cardiac puncture. The abdomen was opened and the islet tumors were decapsulated, weighed, and cut into small pieces for various studies.

Effect of glucose load on serum insulin levels of rats bearing beta cell tumors. Another group of approximately 1-yr-old rats exhibiting persistent hypoglycemia (which were found to have tumors on sacrifice) were lightly anesthetized with ether and bled from the orbital sinus. The animals were injected intravenously with 30% glucose solution (1 ml/100 g body weight) and were bled again 30 min later. The insulin levels were determined in 0.1-ml serum samples using an insulin assay kit ("Phadebas Insulin Test" from Pharmacia AB, Uppsala, Sweden).

Insulin biosynthesis in vitro. Decapsulated tumor samples were cut into approximately 2-mg pieces, and pooled samples weighing between 10 and 40 mg were incubated with shaking (30 oscillations per min) at 37° for 1 or 3 hr in media containing either 60 (3.3 m*M*) or 300 mg/100 ml (16.5 m*M*) glucose, and L-[U-^{14}C]leucine, 311 mCi/mmole, 48 μmole/liter (New England Nuclear Inc.). The medium consisted of 1 ml of Krebs Ringer bicarbonate buffer (KRB) with bovine serum albumin (200 μg), ovomucoid trypsin inhibitor (1 μg), and 19 common amino acids excluding leucine (20 μg each), and was equilibrated with 95% O_2–5% CO_2 gas mixture. Following incubation, tissue and medium were homogenized in the cold in AA glass Teflon grinders (Kontes Glass Co., Vineland, N.J.). Trichloroacetic acid (TCA) was added to a concentration of 10%, and the insoluble material was separated by centrifugation and washed twice with 10% TCA. Insulin and proinsulin biosynthesized during incubation were determined as described recently (5).

Elution profiles of bovine serum albumin,

rat proinsulin, and bovine insulin were used to identify radioactive proteins in column eluates. Ten-microliter aliquots of 1-ml column fractions were assayed for immunoreactive insulin by a double antibody method (6), using rat insulin standards. Protein contents were estimated by the method of Lowry *et al.* (7).

Results. Glucose tolerance test (GTT). Within 3 weeks the animals treated with nicotinamide and streptozotocin exhibited a marked change in their ability to handle a large glucose load (Fig. 1).

The mean blood glucose level of these animals increased from 61 to 369 mg/100 ml within 30 min following the glucose injection, and was 225 and 96 mg/100 ml 60 and 120 min after glucose load, respectively. By contrast in the control rats an identical glucose load resulted in an elevation of the blood sugar levels from 60 mg/100 ml at 0 hour to 305, 100, and 74 mg/100 ml after 30, 60, and 120 min, respectively. The differences at 30 and 60 min were highly significant ($P < 0.01$). About 8 months after the combined drug treatment a large number of rats exhibited persistent hypoglycemia. The nonfasting blood sugar level of these animals was about 20 to 25 mg/100 ml lower than that of the corresponding controls. At the time of sacrifice (about 10 months after the combined drug treatment) all the animals with abnormal GTT exhibited sizable islet beta cell tumors.

A group of animals exhibiting persistent hypoglycemia (which on sacrifice exhibited beta cell tumors) was administered a glucose tolerance test (GTT), 9 to 10 months after combined drug treatment. As shown in Fig. 2, two distinctly different patterns of blood glucose curves were observed as compared to the control animals. The blood glucose levels of the control rats following the glucose load were 83, 299, 126, and 111 mg/100 ml at 0, 30, 60, and 120 min, respectively. By contrast, one group of animals exhibited a tolerance curve typical of a mildly diabetic or subdiabetic animal. The corresponding blood glucose values were 55, 386, 287, and 114 mg/100 ml. The difference from the control rat was highly significant ($P < 0.005$) at 60 min. The beta cells in the islet tumors of these animals perhaps responded sluggishly to the glucose load and hence they were termed "slow acting tumors" (SAT).

The blood sugar levels of the other group were 60, 135, 86, and 77 mg/100 ml, respectively, following glucose administration. The blood sugar levels at 30 and 60 min were markedly lower than those of the

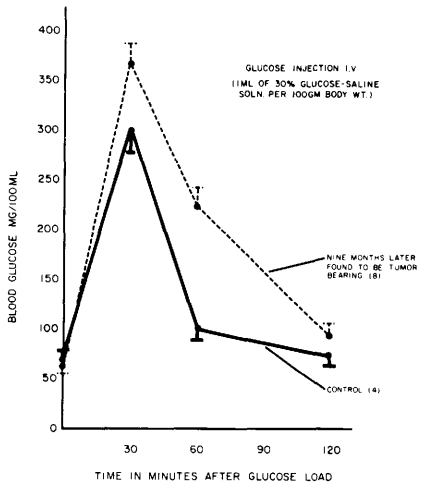

FIG. 1. Abnormal glucose tolerance in rats 3 weeks after treatment with streptozotocin and nicotinamide; these animals exhibited beta cell tumors after 9 months. The differences in the blood sugar levels at 30, 60, and 120 min between the control and drug-treated group were significant, $P < 0.01$, < 0.0001 and < 0.05, respectively.

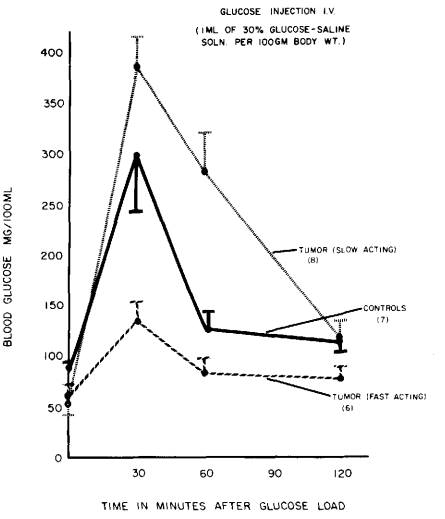

FIG. 2. A comparison of glucose tolerance between normal rats and those bearing beta cell tumors as confirmed by autopsy.

normals at corresponding time periods ($P < 0.005$ and <0.02, respectively).

Immuno-Reactive-Insulin (IRI) in the serum of rats with beta cell tumors. Table I shows that sera collected from rats exhibiting tumors at autopsy could be classified into two distinct categories on the basis of the immunoreactive insulin content (IRI). One group had high IRI content, while in the other the IRI level was normal.

Effect of glucose load on serum insulin levels of beta cell tumor-bearing rats. Table II indicates that the average serum insulin level of rats was elevated from 44 to 73 μU/ml within 30 min following glucose administration. Only three animals failed to show a positive response to the glucose load. At autopsy all these animals exhibited tumors and, hence, data on these three animals have been included for averaging in the table. By contrast, the serum insulin level in five normal controls was elevated from 29 to 38 μU/ml.

Insulin biosynthesis in vitro. The distribution of immunoreactive insulinlike activity following gel filtration is depicted in Fig. 3A. The major insulin region (I) corresponds to a material having a molecular weight of approximately 6000 daltons (monocomponent insulin), while the secondary region (proinsulin) of approximately 9000 daltons had less immunoreactive insulinlike activity. Following 1 hr of incubation, radioactivity was incorporated into the proinsulin (P) region, while none appeared in the insulin region (data not shown). After 3 hr of incubation, some radioactivity also appeared in the immunoreactive insulin region (Fig. 3B). Further evidence for the biosynthetic relationship of these radioactive proteins was shown by tryptic hydrolysis of the isolated proinsulin to material migrating with monocomponent insulin (Fig. 3C). Polyacrylamide gel electrophoresis of the isolated and separated P and I pools of gel filtration eluates showed that the major radioactive protein of the proinsulin (P) region migrated with an R_f of 0.60, while the radioactivity of the insulin (I) region is represented by two (more electronegative) proteins with R_fs of 0.71 and 0.90 (data not shown).

Incubation of tumor tissue in the medium containing 300 mg/100 ml glucose stimulated the incorporation of leucine into both proinsulin and insulin regions of gel filtration eluates, when compared with incubation in medium containing 60 mg/100 ml glucose (Fig. 3B). An experiment involving three adenomata derived from different animals, in which samples of each tumor were incubated at either high or low glucose concentration, is summarized in Table III. Although there was considerable variation in rates of basal and glucose-stimulated leucine incorporation into proinsulin and insulin in the three adenomata, all of them exhibited a response to elevated glucose levels.

Discussion. Multiple pancreatic islet cell tumors were produced in approximately 85% of the rats which survived 8 months or more after the administration of streptozotocin and nicotinamide. This figure compares favorably with the 64 and 49% rates reported by earlier workers (1, 8). Some animals exhibited hypoglycemia within 8 months following drug administration indicating that islet cell tumors may already have been induced before that time.

TABLE I. SERUM IMMUNO-REACTIVE INSULIN (IRI) IN THE RATS WITH BETA CELL TUMORS.

High IRI group		Normal IRI group	
Number of rats	μunits/ml SEM	Number of rats	μunits/ml SEM
6	124 ± 12.7 (76 to 158)	9	40 ± 2.8 (27 to 50)

TABLE II. EFFECT OF GLUCOSE ADMINISTRATION TO TUMOR BEARING AND CONTROL RATS ON THE SERUM IMMUNO-REACTIVE INSULIN (IRI) LEVELS.

		Insulin per ml serum following glucose injection (1 ml of 30% glucose solution per 100 g body weight)			
Condition of rats	Number	0 Min μU/ml SEM	30 Min μU/ml SEM	Difference	P
Tumor	15	44.0 ± 4.3	72.6 ± 9.0	28.6	<0.005
Control	5	29.4 ± 3.3	38.0 ± 5.4	8.6	N.S.

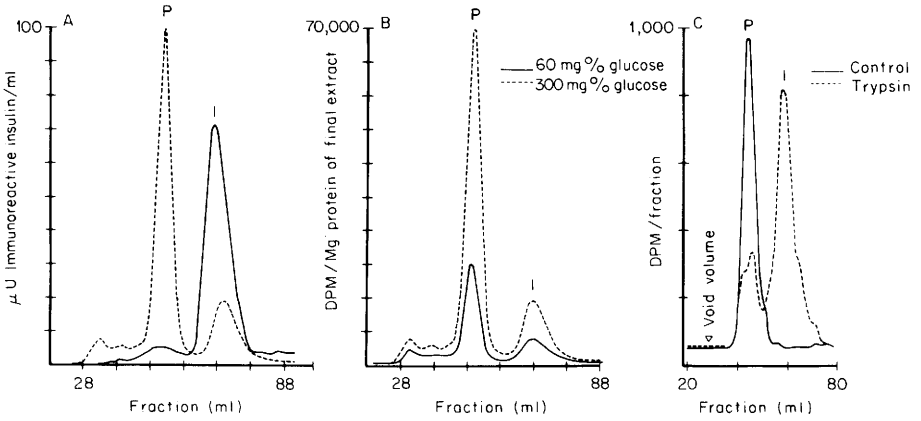

FIG. 3. (A) Immunoreactive insulin in gel filtration eluate of rat adenoma, solid line. Final Extract (1:40,000 dilution). Tissue was incubated 3 hr in KRB containing 300 mg/100 ml glucose. The dashed line represents the incorporation of [^{14}C]leucine into proteins of the same Final Extract. P = proinsulin region of eluate; I = insulin region. (B) Representative gel filtration eluates of Final Extracts from Adenomas incubated in either 60 or 300 mg/100 ml glucose. (C) The effect of trypsin (1 μg) on isolated proinsulin (P) region (about 1 mg protein) of an adenoma incubated with [^{14}C]leucine. The control was an equal aliquot of isolated P which received no tryspin treatment. The void volume, P and I regions were determined by calibration of columns with appropriate standards.

TABLE III. *In vitro* L-[^{14}C]LEUCINE INCORPORATION INTO PROINSULIN (P) AND INSULIN (I) BY RAT ISLET ADENOMA IN THE PRESENCE OF LOW (60 mg/100 ml) OR HIGH (300 mg/100 ml) GLUCOSE IN THE MEDIUM.[a]

	60 mg/100 ml glucose			300 mg/100 ml glucose		
	P	I	I/P	P	I	I/P
Adenoma 1	57,000	26,500	0.47	187,000	61,800	0.33
Adenoma 2	73,800	28,900	0.39	119,000	52,700	0.44
Adenoma 3	346,000	38,300	0.11	681,000	89,000	0.13

[a] Tissue pieces from three adenomas were incubated concurrently for 3 hr in KRB with either the low or high glucose concentration, and extracted in parallel. Results are expressed as disintegrations per minute per milligram of protein of the final extract.

The abnormal glucose tolerance curve 3 weeks after the treatment with the combined drugs, may indicate that although the animals did not exhibit manifest diabetes, they were unable to handle a sudden large load of glucose. The DI of these rats was 2.92. According to Coupland *et al.* (4) a DI in excess of 1.7 is indicative of subdiabetic state. All the rats with subdiabetic DI developed pancreatic islet tumors. This observation suggests some relationship between chronic exhaustion of the beta cell and its hyperplasia and eventual tumor development.

Animals whose glucose tolerance indicated that they had slow acting tumors had a DI of 2.34 while animals with fast acting tumors had a DI of 0.47.

The serum immunoreactive insulin levels in rats with tumors were either high (124 μU/ml) or normal (40 μU/ml) (Table 1). On the basis of serum IRI levels the rats could not be designated as containing fast or slow type of tumors. All the rats with tumors were capable of responding to increased blood glucose levels as shown by the fact that their insulinogenic response to glucose was greater than that of the corresponding control rats (Table 2). The elevation of serum IRI level in controls at 30 min following glucose administration was not significant. Perhaps IRI measurements at earlier time periods may have been more meaningful.

Insulin biosynthetic activity of induced adenomata was demonstrated by means of the *in vitro* incorporation of [^{14}C]leucine into proinsulin and insulin. [^{14}C]proinsulin was readily converted to an insulinlike molecule by trypsin treatment (10, 11). The

migration of these proteins on gel electrophoresis closely paralleled those isolated from islets of Langerhans obtained from normal rats by the collagenase digestion procedure (5).

On the basis of radioimmunoassay, monomeric (6000 daltons) insulin represented the major type of immunoreactive insulin in one adenoma (Fig. 3A). Protein material migrating in the 9000-dalton range mostly consisted of proinsulin, and accounted for less than 10% of the immunoreactive insulin in this adenoma. Gel filtration eluates of two other adenoma extracts were also assayed and showed essentially the same pattern of immunoreactivity.

Creutzfeldt *et al.* (11) reported an average tumor insulin content in human adenomata of 24 U/g (0.01–199 U/g). In the rat adenomata reported here, the average IRI content was 412 U/g (165–729 U/g) suggesting that the tumors have large stores of insulin. By contrast, normal rat islets contain 200 U/g of insulin (12).

The ratio of [^{14}C]insulin to [^{14}C]proinsulin, which estimates the extent of conversion of precursor to insulin, was the same at basal (60 mg/100 ml) and elevated (300 mg/100 ml) glucose concentrations, suggesting that enzymatic conversion of proinsulin to insulin is not a limiting step in insulin production by the induced adenomata. In all tumor studies, the elevated level of glucose in the medium (300 mg/100 ml) caused an increased rate of [^{14}C]leucine incorporation into (pro)insulin when expressed on the basis of specific activity (disintegrations per minute per milligram of protein in the extract).

Summary. Islet beta cell adenomata were induced in rats by combined treatment with nicotinamide and streptozotocin. Three weeks after treatment marked alterations in glucose tolerance were noted in animals which later exhibited large beta cell tumors. Eight months after treatment, the rats known to have beta cell tumors on the basis of marked hypoglycemia and later confirmed by autopsy showed variable response to a glucose load. Some tumor-bearing rats showed fast response to glucose load, their blood sugar levels were elevated moderately and returned to normal or below normal levels rapidly; these animals are described as having "fast-acting tumors." Rats with "slow-acting tumors" responded sluggishly to a glucose load; their blood glucose pattern was similar to that of subdiabetic animals. Animals with beta cell tumors exhibited elevated serum insulin levels 30 min after glucose administration.

Insulin biosynthesis by beta cell adenomata was demonstrated by *in vitro* incorporation of [^{14}C]leucine into proinsulin and insulin. In the small number of tumor samples studied, a stimulatory effect of glucose on insulin biosynthesis was observed.

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This paper is dedicated to the memory of the late Dr. Arnold Lazarow.

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