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ORIGINALS

Further Studies on the Amelioration of the Characteristics of New Zealand Obese (NZO) Mice Following Implantation of Islets of Langerhans

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Summary. New Zealand Obese (NZO) mice are characterised by hyperglycaemia, hyperinsulinaemia and obesity. The blood glucose and plasma insulin levels of these mice can be lowered to levels approaching those normally associated with non-obese mice, by the intraperitoneal implantation of pancreatic islets from albino mice which have had their β-cells selectively destroyed by streptozotocin. Removal of these implanted islets causes the animals to revert to their original state. It would seem, therefore, that the genetic lesion responsible for the NZO state lies within the islets of Langerhans but not within the beta-cell. Its exact location is as yet unknown.

Key words: Islet implantation; streptozotocin; reversion of NZO mice

We have previously reported [1] that when NZO mice were implanted with islets isolated from normal albino mice, their weight gain was reduced and blood glucose and plasma insulin levels significantly lowered. This supported the suggestion of Strautz [2] that the spontaneous obesity which develops in the genetically obese mouse results from missing or defective pancreatic factor/s present within the islets of Langerhans. Our present studies are an attempt to identify further the cells responsible for the secretion of this factor.

Materials and Methods

Source and Care of Mice

The NZO mice were derived from the original stock of Bielschowsky and Bielschowsky [3] and bred, by brother/sister mating, in our own unit. The albino mice were supplied by Evans, Carshalton, Surrey, England (from Medical Research Council stock). All adult animals were housed in metal cages and given free access to food (PRD cubes, Christopher Hill Group) and tap water.

Islet Isolation and Implantation Procedure

Islets were isolated by a collagenase technique, sealed in diffusion chambers constructed from nylon-reinforced Millipore membranes and implanted intraperitoneally as described elsewhere [1].

Up to 3,000 islets could be collected in one day and 16 animals implanted. The usual number implanted in one day was 12.

The NZO mice were male, aged 5 to 8 weeks and separated into four groups, A, B, C and D. Following initial weighing, blood was sampled from the clipped tail of each animal for glucose and insulin determination, after which each mouse received intraperitoneal implants of Millipore diffusion chambers containing either 200 islets isolated from normal albino mice (Group A), 200 similar islets but exposed to streptozotocin (Group B), chambers with no tissue though treated with streptozotocin (Group C) or chambers with no tissue (Group D).

Streptozotocin Treatment of Islets

Approximately 200 islets were sealed in diffusion chambers and placed in a 300 mg% solution of streptozotocin (Upjohn & Co.) pH 4.5 as described by Junod *et al.* [4]. The chambers were then agitated in a water bath maintained at 38°C for four minutes. Following this period of incubation, the chambers were transferred to Gey and Gey Ringer solution containing 30 mg% glucose and agitated for a further 20 min to remove residual traces of streptozotocin. Some chambers containing islets were subjected to the same procedure but in the absence of the cytotoxic agent (Group A).

Implant Removal

Implanted diffusion chambers were surgically removed from four animals of Group B and for a period of 6 weeks the body weight, blood glucose and plasma insulin levels were monitored.

Isolated Islet Function Tests

The functional activity of the islets both before and after implantation was examined by the sequential placing of the sealed chambers containing the islets in known volumes of Ringer solution containing the agents under study. Additionally, normal and streptozotocin-treated islets were tested, after a 6 week period of implantation, for their ability to synthesise proinsulin and insulin. Incorporation of ^{3}H-leucine into these hormones was determined by a modified method of Schatz *et al.* [5]. Following removal from the mice the chambers were incubated in the presence

of L-leucine-4,5-^{3}H (50 μCi/ml, 30—50 Ci/mmole, New England Nuclear).

Body-Weight, Blood Glucose and Plasma Insulin Determination

All mice were weighed and blood samples taken from the tail at 0, 5, 15, 25, 35 and 45 days following implantation. Glucose was estimated by a modification of the Faulkner [6] method using a continous-flow analyser and plasma insulin by a double-antibody technique [7] using ox insulin as a standard.

Results

Minimum Number of Islets Necessary to Cause a Reversion to the Non-obese State

In order to find the minimum number of islets necessary to cause reversion, NZO mice were implanted with Millipore chambers containing 150, 100, 50 and 25 albino islets. Fig. 1 and 2 show that 50 is the least

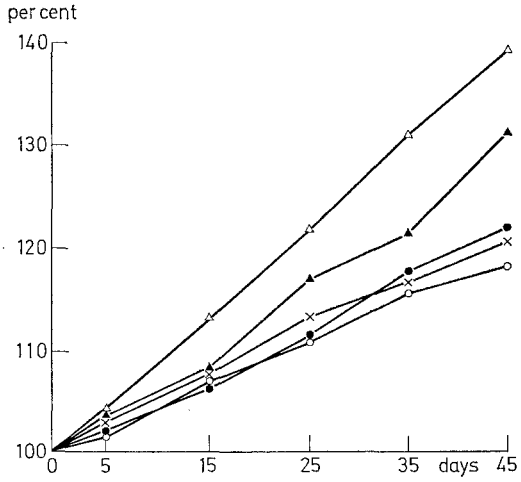

Fig. 1. The % change in body weights of NZO mice when implanted with varying numbers of isolated albino islets. The number of animals per group is shown in parenthesis. Figures refer to pre- and post-implant values respectively. SEM are shown. Weight in grams.

—●—●—	150 islets (8)	24±0.3	30±0.4
—○—○—	100 islets (7)	24±0.5	29±0.6
—×—×—	50 islets (9)	24±0.2	29±0.3
—▲—▲—	25 islets (7)	25±0.7	32±0.8
—△—△—	Control (9) (chamber only)	25±0.4	35±0.6

Body weight changes in the group having received 50, 100 and 150 islets were significantly different from that of the control group ($p > 0.001$). That of 25 islets ($p > 0.05$)

number necessary for complete reversion, 25 islets seem to cause partial reversion. Implantation with obese islets, pancreatic acinar tissue, liver and muscle showed that reversion was obtained only with implantation of albino islets thus eliminating both the exocrine pancreas and allergic reactions as being responsible for the return to normal.

Streptozotocin-Treatment of Isolated Islets

In order to verify that streptozotocin had inactivated the β-cell of the pancreatic islets, Millipore chambers of Group B were incubated in alternate high and low concentrations of (a) glucose and (b) arginine (in the presence of 30 mg% glucose). Table 1 shows that untreated islets release insulin appropriately when challenged with either arginine or glucose whereas streptozotocin-treated islets do not release insulin in amounts proportional to the secretagogue present. The pattern suggests a washing out of residual insulin following β-cell destruction. After implantation, no insulin is released from the streptozotocin treated islets. Table 2 shows that islets maintain their integrity and function following 4 min incubation in a buffer at pH 4.5, but in the absence of streptozotocin.

Fig. 3 (a) shows that after the implantation term, the islets exposed to streptozotocin do not incorporate ^{3}H-leucine into either insulin or proinsulin whereas Fig. 3 (b) shows that islets not exposed to streptozotocin and implanted for a similar period do incorporate ^{3}H-leucine into both these hormones.

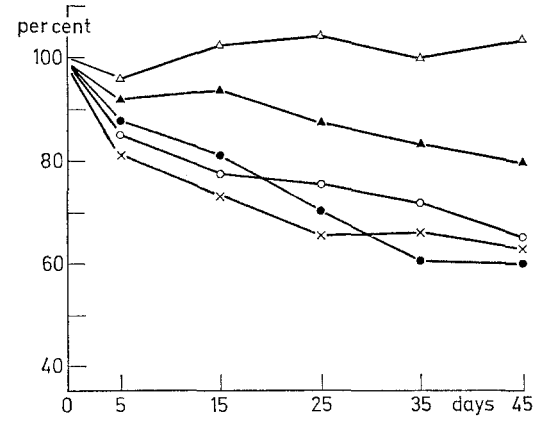

Fig. 2. The % change in blood glucose levels of NZO mice when implanted with varying numbers of isolated albino islets. See legend of Fig. 1 for further details.

—●—●—	150 islets (8)	187±3.7 to 110± 7.1
—○—○—	100 islets (7)	191±3.3 to 125± 7.5
—×—×—	50 islets (9)	201±5.7 to 126± 4.7
—▲—▲—	25 islets (7)	211±8.3 to 173±18.0
—△—△—	Control (9) (chamber only)	186±6.2 to 190± 7.9

Blood glucose changes in the group having received 150, 100 and 50 islets were significantly different from that of the control ($p > 0.001$). That of 25 islets ($p > 0.05$). Glucose values in mg%

In addition when implanted chambers were removed from animals of Group B, macerated and then tested for insulin content, the total amount found in the original 200 islets was less than 15 μU, which probably represents the concentrations of insulin present in physiological fluids. 25 untreated islets subjected to the same procedure gave an insulin content of 176 μU. Thus, untreated islets contained approximately 100 times as much insulin as streptozotocin treated islets.

These findings show that both the secretory as well as the synthetic functions of the β-cell have been destroyed by exposure to streptozotocin.

The results summarised in Figs. 4 to 6 are based on observations made on 38 animals that received implantation of Millipore diffusion chambers. Each point represents the arithmetic mean of group samples.

Body weight gains in the control groups (C and D) were greater than those found in animals having received implants containing either normal or streptozotocin-treated albino islets (Groups A and B), Fig. 4.

There was no significant change in blood glucose in the control groups over the 45 day experimental period whereas the levels of Groups A and B showed a significant decrease compared to Groups C and D, Fig. 5.

The plasma insulin levels showed a trend similar to that of the blood glucose pattern. The levels in Groups A and B decreased significantly from those of the control groups (C and D) whose hyperinsulinaemia remained constant over the 45 days. Fig. 6.

Table 1. *Arginine and glucose mediated insulin release from chambers containing untreated and streptozotocin-treated islets*

	Arginine Concentration (in the presence of 30 mg% glucose)				
	5mM	0.5 mM	5 mM	0.5 mM	5 mM
Untreated islets (not implanted)	125 220	45 82	93 147	73 62	115 143
Untreated islets after 45 days implantation	80 63	37 28	94 112	57 40	107 93
Streptozotocin-treated islets prior to implantation	154 162 101 86	47 72 44 12	70 55 44 8	80 73 60 26	74 63 53 22
Streptozotocin-treated islets after 45 days implantation	0 0 0 0	0 0 4 0	4 0 0 0	0 8 0 0	7 0 5 0
	Glucose Concentration				
	300 mg%	30 mg%	300 mg%	30 mg%	300 mg%
Untreated islets (not implanted)	280 186	150 83	188 172	116 102	205 214
Untreated islets after 45 days implantation	77 130	42 68	108 123	56 79	120 142
Streptozotocin-treated islets prior to implantation	73 13 26	15 10 0	0 6 0	0 12 0	0 0 8
Streptozotocin-treated islets after 45 days implantation	0 0 0	6 0 10	0 0 10	5 0 0	0 0 0

Figures are μU insulin released per ml of incubate per 10 min

Table 2. *Normal islets incubated for 4 min at pH 4.5 and then stimulated with high (300 mg%) and low (30 mg%) glucose*

H	198	288	157	247
L	52	86	92	63
H	320	192	206	184
L	27	23	115	96
H	167	72	198	222

Figures are μU insulin released per ml incubate per 10 min

Effect of Removal of Implanted Chambers on the Body Weight, Blood Glucose and Plasma Insulin

Diffusion chambers containing streptozotocin-treated islets were surgically removed from four mice of Group B after the experimental term of 45 days. During the next six weeks, the animals reverted to the obese state with attendant hyperglycaemia and hyperinsulinaemia, Fig. 7.

Discussion

When NZO mice are implanted intraperitoneally with Millipore diffusion chambers containing islets isolated from normal albino mice the hyperglycaemia

and hyperinsulinaemia, associated with NZO mice, return to levels similar to that of non-obese mice [1]. This finding indicates that the genetic lesion responsible for the NZO state lies within the islets of Langerhans.

We have demonstrated that this effect is specific for normal islets in that NZO islets, acinar tissue, liver or muscle did not cause reversion. The minimum number of islets necessary for reversion to take place was found to be 50.

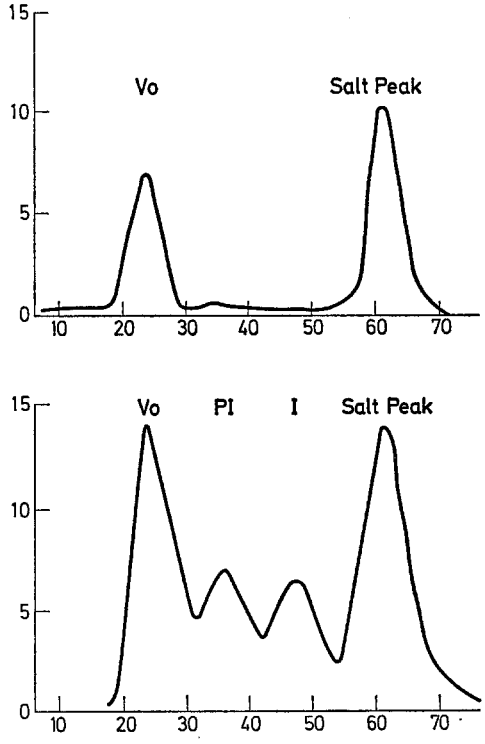

Fig. 3. Incorporation of ^{3}H-leucine into proinsulin and insulin. See text for method. Chambers of 200 islets used 45 days following implantation. a) Streptozotocin treated islets. There is no incorporation of leucine into either proinsulin or insulin. b) Untreated islets, leucine is incorporated into both proinsulin and insulin. Vo = void volume. PI = proinsulin. I = insulin. Ordinate = cpm × 10^3. ABSCISSA = fraction number

It has been shown that there are many cell types present in the islet. In addition to insulin, glucagon and gastrin secreting cells, a fourth cell type has recently been identified [8]. The functional significance of this cell is unknown. In order to narrow the field we investigated the effect of implanting streptozotocin-treated islets. It was found that harvesting islets from streptozotocin-induced diabetic mice was difficult due to islet fragility. An *in vitro* technique was devised. This technique efficiently destroyed the β-cell as shown by the following evidence. The streptozotocin-treated islets were unable to elicit the proper insulin response when placed sequentially in high and low concentrations of either arginine in 30 mg% glucose or glucose. Evidence of destruction was emphasized by the fact that the islets released no insulin following removal of the implant from the animal after 6 weeks. In addition, when implanted chambers were removed from the animal, macerated and then tested for insulin content only 15 μU was found in 200 islets. This amount was 100 times less than that found in untreated islets. Streptozotocin-treated islets were unable to incorporate ^{3}H-leucine into either proinsulin or insulin.

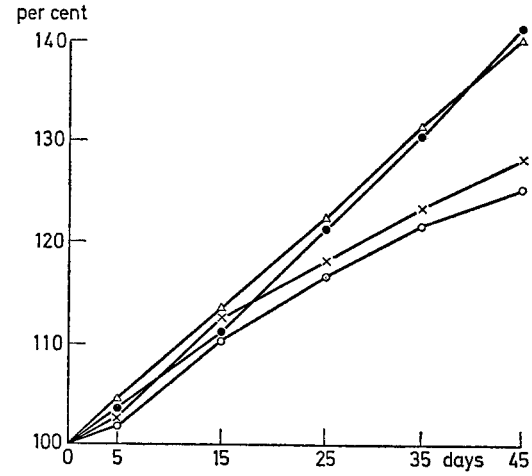

Fig. 4. The percentage change in body weight of NZO mice following implantation of streptozotocin-treated albino islets. Number of animals in groups shown in parenthesis.

—○———○— Group A (8) chamber with untreated albino islets. Mean increase of 26% (23 g±0.7 to 29 g±0.4).

—×———×— Group B (10) chamber with albino islets treated with streptozotocin. Mean increase of 30% (23 g±0.6 to 30 g±0.5)

—●———●— Group C (9) chamber only, treated with streptozotocin. Mean increase of 41% (22 g±0.5 to 31 g±0.4)

—△———△— Group D (9) chamber only, untreated. Mean increase of 39% (23 g±0.5 to 32 g±0.4)

No significant difference between Groups A and B.
No significant difference between Groups C and D.
Group B is significantly different from Group C ($p<0.001$)
Group A is significantly different from Group D ($p<0.001$)
Group B is significantly different from Group D ($p<0.001$)
Group A is significantly different from Group C ($p<0.001$)

Streptozotocin-treated islets are thus capable of returning the weight gain, the blood glucose and plasma insulin of NZO mice to levels normally associated with albino mice. We conclude from this that insulin does not play a role in the reversion process.

These results are in agreement with those obtained by Strautz [2] using ob/ob mice. This suggests that the symptomatology exhibited by both NZO and ob/ob mice arises from the lack of the same substance or substances. We would suggest that there is more than one gene controlling the mechanisms necessary to

produce and secrete this unknown substance and that the different hereditary characteristics shown by these two species are a reflection of the lesions.

More intriguing at the moment is the possible mode of action of this unknown substance/s. Mahler and Szabo [9] have shown that it is possible to get reversion of ob/ob characteristics by controlled treatment of suggest that the change in peripheral receptors could occur as a result of hyperinsulinaemia, reinforcing the previous suggestion that hypersecretion of insulin may be a primary fault. It has been shown that there is a close relationship between the functioning of α- and β-cells in the islets [12]. It would seem logical to extend this finding and postulate that the missing

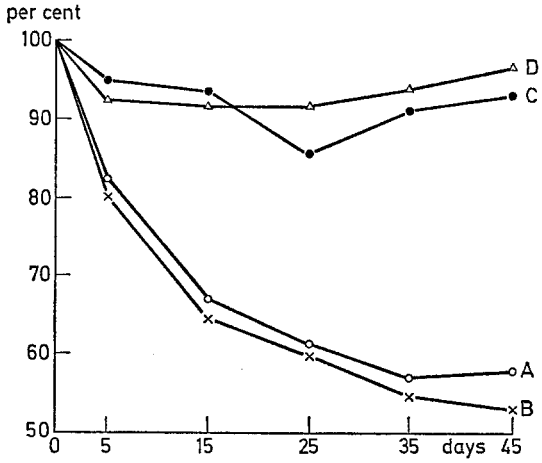

Fig. 5. The percentage changes in blood glucose levels of NZO mice following implantation of streptozotocin treated albino islets. See Fig. 4 for description of groups

—○———○— Group A. Mean decrease of 43% (208±2.8 to 118±3.4 mg per 100 ml)

—×———×— Group B. Mean decrease of 49% (221±3.5 to 113±1.6 mg per 100 ml)

—●———●— Group C. Mean decrease of 8% (194±2.2 to 179±1.8 mg per 100 ml)

—△———△— Group D. Mean decrease of 3% (192±3.1 to 187±1.2 mg per 100 ml)

Group (A) is not significantly different from Group (B)
Group (A) is significantly different from Group (C) ($p<0.001$).
Group (A) is significantly different from Group (D) ($p<0.001$).
Group (B) is significantly different from Group (C) ($p<0.001$).
Group (B) is significantly different from Group (D) ($p<0.001$).
There was no significant difference between Groups (C) and (D)

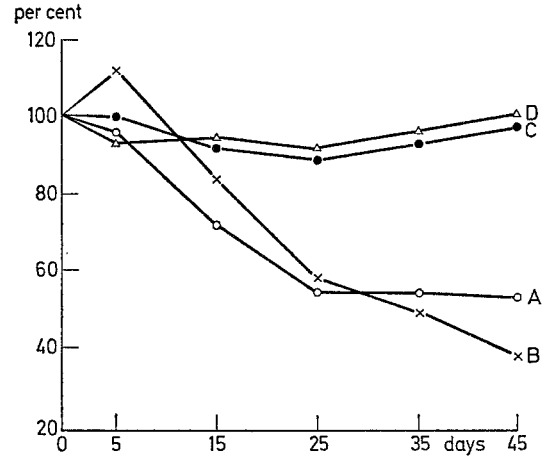

Fig. 6. The percentage changes in plasma insulin levels of NZO mice following implantation of streptozotocin treated albino islets. See Fig. 4 for description of groups.

—○———○— Group A. Mean decrease of 43% (192±4.6 to 104±7.3 μU per ml).

—×———×— Group B. Mean decrease of 61% (168±5.0 to 66±3.2 μU per ml).

—●———●— Group C. Mean decrease of 3% (168±4.2 to 163±4.1 μU per ml).

—△———△— Group D. Mean decrease of 1% (175±3.5 to 173±2.4 μU per ml).

Group (A) is not significantly different from Group (B).
Group (A) is significantly different from Group (C) ($p<0.001$).
Group (A) is significantly different from Group (D) ($p<0.001$).
Group (B) is significantly different from Group (C) ($p<0.001$).
Group (B) is significantly different from Group (D) ($p<0.001$).
There was no significant difference between Groups (C) and (D)

the β-cell with alloxan, and have suggested that hypersecretion of insulin is the primary lesion in these animals. Kahn *et al.* [10] and Baxter, Gates and Lazarus [11] have reported that there is a deficiency of insulin receptors in ob/ob and NZO mice respectively. Baxter, Gates and Lazarus [11] found that when NZO mice were reverted by implantation the number of insulin receptors in the liver increased until the number was identical to that found in white mice. However, the increase in receptors lagged well behind the decrease in both plasma insulin and blood glucose. These results factor in both NZO and ob/ob mice also plays a role in the normal integrated function of the islet. We suggest that one of the actions of this unknown substance is to regulate insulin secretion.

We speculate whether there may not exist in man an obese state that is due to a partial or total lack of this factor. The attendant hyperinsulinaemia would then produce a diminution in the number of insulin receptors with consequent dislocation of glucose metabolism and the appearance of all those symptoms associated with insulin resistance.

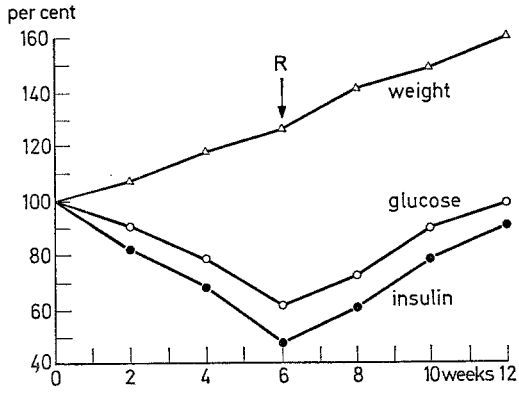

Fig. 7. The effect on blood glucose, plasma insulin and body weight of the removal of implanted streptozotocin treated albino islets.
R = time of implant removal. Points are arithmetic means of 4 mice.

—△———△— Body weight. Initial values of 23 g, 30 g at time 'R', 39 g after a further 6 weeks.

—○———○— Blood glucose. Initial values of 218 mg%, 113 mg% at time 'R', 208 mg% after a further 6 weeks.

—●———●— Plasma insulin. Initial values of 169 μU/ml, 66 μU/ml at time 'R', 145 μU/ml after a further 6 weeks.

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