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CHANGES IN THE DIABETIC STATE BROUGHT ABOUT BY THE TRANSPLANTATION OF EMBRYONIC PANCREATIC TISSUE TO ALLOXAN-DIABETIC MICE

BY

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CHANGES IN THE DIABETIC STATE BROUGHT ABOUT BY THE TRANSPLANTATION OF EMBRYONIC PANCREATIC TISSUE TO ALLOXAN-DIABETIC MICE

by

Walter W. Karney

A Thesis Presented to the Faculty of the Yale University
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1962

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INTRODUCTION

An insufficient supply of insulin within the body results in the alteration of certain metabolic processes which, if uncontrolled, progresses to the chronic disease state of diabetes mellitus. These multiple metabolic disturbances can be controlled by the adequate administration of exogenous insulin. It would be of importance to know whether these metabolic derangements also could be controlled by incorporating within the body a continuous source of insulin in the form of transplants of pancreatic tissue.

Various techniques have been devised to create artificially a diabetic animal on which a variety of studies can be conducted. Of these methods, the administration of alloxan has proved to be a relatively expedient and reliable procedure. Dunn and co-workers in 1943 discovered that alloxan, which is the ureide of mesoxalic acid, when given parenterally to the rabbit caused selective acute necrosis of islet cell tissue[1]. The injection of alloxan is followed immediately by an initial hyperglycemia lasting 15 minutes to 1 hour. Subsequently there is a marked hypoglycemia apparently caused by insulin released from damaged islet cells. Three to 5 hours after the injection, hyperglycemia and permanent diabetes ensue. Concerning the possible action mechanism of alloxan, it has been found that after its administration the glutathione concentration normally present in the blood is greatly reduced. Moreover, the destruction of islet cells by alloxan can be protected against by the administration of glutathione in large doses[2].

An experiment designed to investigate whether pancreatic tissue transplanted to a diabetic host can alter the diabetic state was carried out in 1951 by Browning and Resnik[3]. They were interested in the fate and physiologic

function of embryonic and neonatal pancreatic tissue in the intraocular, subcutaneous, and subcapsular splenic sites of diabetic hosts. The host animals were made diabetic using intraperitoneal alloxan, 175 mg in 1% aqueous solution per Kg. In the control group (no transplant) all the mice were dead by the 35th day post alloxan. The other mice received one of the following types of transplants: 1. One embryonic pancreas intraocular to both eyes, 2. Two embryonic pancreata subcutaneously, 3. Two embryonic pancreata to the subcapsular splenic site, 4. Two embryonic pancreata intraocular to both eyes, 5. Two newborn pancreata subcutaneously, or 6. Four newborn pancreata to the subcutaneous space. The only significant effect on the diabetes was seen in the mice which had received 4 newborn pancreata in the subcutaneous space. In these animals the glycosuria had decreased by the 30th day, and they were still alive on the 90th day. Most of the other mice were dead by the 45th day with marked glycosuria. It was found that the embryonic tissue grew and differentiated, but that the newborn tissue differentiated without growth. There was development of vascularization, cysts, opacities and fat. Good differentiation and organization of islet cell tissue occurred, but there was only poor representation of alveolar components. Embryonic transplants grew to 1.5 to 3 times their original surface area. The development of the grafts in homologous hosts was the same in the intraocular and subcutaneous sites; however, the subcapsular splenic transplants did not persist.

In reviewing these results, it should be pointed out that the alloxan diabetes produced by Browning and Resnik was always severe (3+glycosuria), and that there was no way of telling whether a mild diabetic state might

have been controlled by smaller amounts of pancreatic tissue. In this regard, it has been found that the susceptibility to alloxan of animals of different species, and even of different strains of the same species, varies greatly. This differential susceptibility to alloxan of various strains of mice, and the dosage of alloxan required to produce diabetes in the different strains was clearly elucidated by Martinez et al. in 1954.[4] It was determined that in most strains an intravenous injection of approximately 70 mg per Kg of alloxan monohydrate is sufficient to produce a mild diabetic state. Furthermore, Browning and Resnik never showed that the diabetic condition produced by alloxan was being controlled exclusively by the tissue transplants. In order to prove this conclusively it would be necessary to surgically remove the transplanted pancreas and observe the animal as it reverted to its former diabetic state. It was with these objectives in mind that the following experiment was conducted.

MATERIALS AND METHODS

Two groups of studies were conducted. In the first, 31 adult mice of the dba strain which had been tested for glycosuria and were found to have less than 0.1% on two occasions were given i.v. alloxan 70 mg per Kg into the tail vein. They were all subsequently shown to be diabetic. The degree of glycosuria was the criterion used to estimate the severity of the diabetic state, and the Denco sugar test, a modification of the Nylander test was employed[5]. The results of this method correlated exactly with those obtained using Clinitest as a reagent in a controlled experiment, and the Denco test has the advantage of requiring only a single drop of urine for accurate results. In order to collect the urine, an animal was first injected with

1 ml of water intraperitoneally and then placed inside a large funnel set into a test tube into which the urine passed. At the end of 1 hour there was usually sufficent urine for test purposes.

Within 1 week post alloxan, 16 of the 31 mice had died, presumably from an ectomelia infection which ran rampant through many of the mice in the animal colony. Of the 14 remaining mice, 4 were kept as controls, having no further procedures done. Ten days after the alloxan injection, a single embryonic dba pancreas in the 16th day of gestation was transplanted to one anterior chamber of 5 adult dba mice which had been anesthetized with nembutal according to the technique described by Greene[6]. Because of the difficulties encountered in acquiring embryonic mouse pancreas in the desired time of gestation, it was possible to obtain only a limited amount of this tissue. Also, any attempt to recover mouse pancreas before the 16th day of pregnancy was met with failure because of the minute size and extreme friability of the tissue. Five other adult dba mice received 2 embryonic dba pancreata into the subcutaneous space of the axilla using a similar technique.

One of the control animals was sacrificed 2 weeks post alloxan to determine the pancreatic histology, and the other 3 were autopsied 135 days after the alloxan injection. In 2 mice whose anterior chambers contained apparently successful transplants, the eyeball was removed and the animals then observed and tested for glycosuria until termination of the experiment 1 month later. Three of the 5 mice which developed palpable tumors in the region of the subcutaneous transplant had this tissue removed operatively and were also observed until the end of the experiment. One-hundred twenty-five days after the transplantation had been done, all remaining mice were

sacrificed and sections taken of appropriate tissue.

In the second series of studies a new group of 30 adult dba mice to be used as host animals were again tested for glycosuria, and found to be negative on two occasions. They were given i.v. alloxan 70 mg per Kg and shortly thereafter were demonstrated to be diabetic. Five animals were kept as controls, and 2 of these were sacrificed to recover the pancreata at different periods post alloxan. Three days after alloxan, 5 of the host mice each received 1 newborn dba pancreas in the 21st day of gestation into the anterior chamber, and 2 of these animals were enucleated about 6 weeks before all of the mice remaining in the experiment were sacrificed. Fifteen subcutaneous transplants were performed. Ten mice each received 2 embryonic dba pancreata in the 16th day of gestation, and 5 each were the recipients of 2 embryonic pancreata of the C_3H strain of mice. Of the 5 animals in the former group who went on to develop small subcutaneous masses, 4 were operated on in an attempt to remove the transplanted tissue. All remaining mice in this second series of studies were sacrificed 111 days post alloxan, and the appropriate pancreatic tissues recovered.

RESULTS

From the summary presented in Table I and Table II it can be seen that 29 of an original group of 61 mice had survived until termination of the experiment. Of the 29, 5 were controlled animals which had lived between 111-135 days after the alloxan injection, and in all probability would have survived longer if it had not been necessary to end the study. Three other control mice (Nos. 1, 54, and 55) were sacrificed during the course of the

TABLE I - RESULTS OF GROUP I

	Mouse No.	Day 1-4	5	8	14	15	18	22	29	43	57	69	76-104	118	125	140	143
Controls	1	0	A	1	2		3	N									
	2	0	A	1	1		2	1	1	1	3	2	1-4	3	2	3	N
	3	0	A	2	1		0	2	1	2	3	1	1-2	2	1	1	N
	4	0	A	1	1		1	2	1	1	2	3	1-2	2	1	2	N
Embryonic dba pancreas to Anterior Chamber	5	0	A	1	2	T	0	0	1	1	2	1	1-2	2	1	2	N
	6	0	A	1	1	T	1	0	1	1	1	2	1 E	2	2	1	N
	7	0	A	1	2	T	2	1	0	0	0	1	1-2 E	3	4	3	N
	8	0	A	1	3	T	2	1	1	0	1	2	1-2	1	2	2	N
	9	0	A	1	1	T	1	1	2	1	3	3	1-2	2	1	2	N
Embryonic dba Pancreas to Subcutaneous Space	10	0	A	1	2	T	2	1	0	0	1	2	1-2	1	1	2	N
	11	0	A	1	1	T	1	1	0	0	0	0	S 1-2	3	3	3	N
	12	0	A	1	2	T	1	1	1	0	0	1	1-2 S	4	2	3	N
	13	0	A	2	2	T	2	1	2	0	0	1	S 1-2	2	3	2	N
	14	0	A	4	1	T	4	4	4	3	4	4	3-4	4	4	4	N
	15-31	0	A	Dead within one week post alloxan													

Less than 0.1% glycosuria = 0
0.1 - 0.3% glycosuria = 1
0.4 - 0.6% glycosuria = 2
0.7 - 0.9% glycosuria = 3
1.0% or greater glycosuria = 4

A = Alloxan injection
T = Transplant performed
E = Enucleation
N = Autopsy
S = Attempt at removal of subcutaneous transplant

TABLE II - RESULTS OF GROUP II

	Day Mouse No.	1-3	4	6	7	10	17	30	38	49	63	70	77	89	105	115
Embryonic dba Pancreas to Subcutaneous Space	32	0	A	2	T	2	0	1	1	0	0	0	1 S	(Died at surgery)		
	33	0	A	1	T	1	0	1	1	0	0 S	0	0	1	1	N
	34	0	A	1	T	0	1	1	2	1	1	1	1	1	1	N
	35	0	A	1	T	1	1	3	1	0	0	0	0	0	0	N
	36	0	A	2	T	2	0	3	1	0	0 S	0	1	1	1	N
	37	0	A	3	T	1	0	2	2	N						
	38	0	A	2	T	2	1	1	2	2	1	1	1	1	1	N
	39	0	A	2	T	2	1	2	3	0	0	1	1	2	1	N
	40	0	A	2	T	0	1	1	2	0	0 S	0	1	1	0	N
	41	0	A	2	T	0	1	3	1	1	0	0	0	1	2	N
Embryonic C_3H Pancreas to Subcutaneous Space	42	0	A	0	T	0	1	2	2	3	2	2	3	2	2	N
	43	0	A	3	T	3	1	4	Dead							
	44	0	A	1	T	0	Dead									
	45	0	A	1	T	1	1	1	1	2	1	2	2	2	3	N
	46	0	A	2	T	0	0	1	N							
Newborn dba Pancreas to Anterior Chamber	47	0	A	2	T	2	1	2	1	2	1	2	2	3	3	N
	48	0	A	1	T	3	Dead									
	49	0	A	2	T	1	1	2	1	2	0	1 E	1	2	2	N
	50	0	A	1	T	1	1	3	1	2	1	0 E	2	1	2	N
	51	0	A	2	T	1	3	4	4	4	4	4	4	4	4	N
Controls	52	0	A	2		1	2	2	1	2	1	1	1	2	2	N
	53	0	A	1		1	1	2	3	2	3	2	3	4	3	N
	54	0	A	3		1	1	N								
	55	0	A	1		2	1	1	3	2	N					
	56	0	A	1		2	1	2	3	3	2	2	3	2	N	
	57-61	0	A	Dead within two days post alloxan												

Less than 0.1% glycosuria = 0
0.1-0.3% glycosuria = 1
0.4-0.6% glycosuria = 2
0.7-0.9% glycosuria = 3
1.0% or greater glycosuria = 4

A= Alloxan injection
T= Transplant performed
E= Enucleation
N= Autopsy
S= Attempt at removal of subcutaneous transplant

experiment. From a glance at Table I and Table II it is evident that all the control mice had severe glycosuria (greater than 2+ or 0.4%) at some time after alloxan, and with the exception of mouse no. 3, all continually had greater than 0.1% glycosuria. The pancreata of all the control mice autopsied revealed essentially the same changes as those seen in Figure I and Figure II which are taken from the pancreas of mouse no. 53. There is karyolysis and dropping out of cells producing a relative scarcity of islet cells.

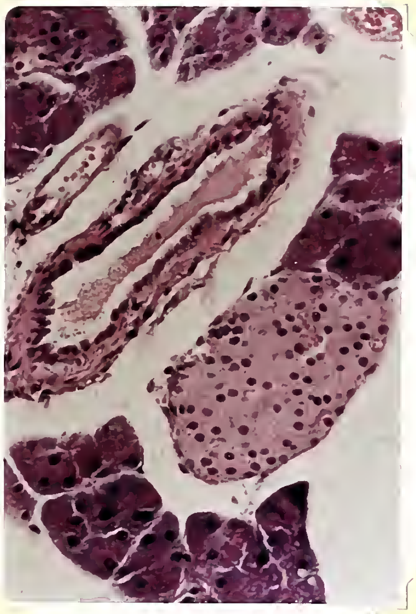

Fig. I from mouse no. 53
(x250)

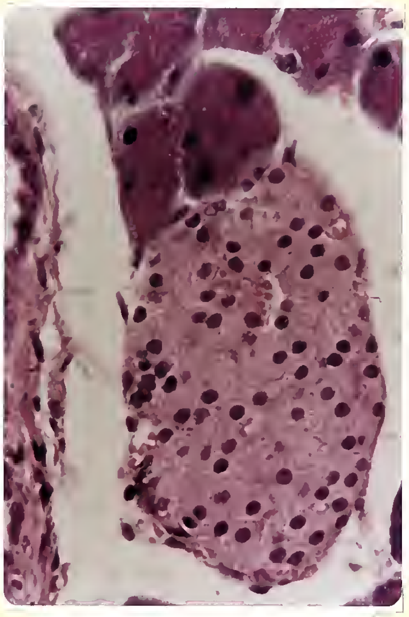

Fig. II from mouse no. 53
(x400)

For comparison, an islet from a normal mouse pancreas is shown in Fig. III and IV.

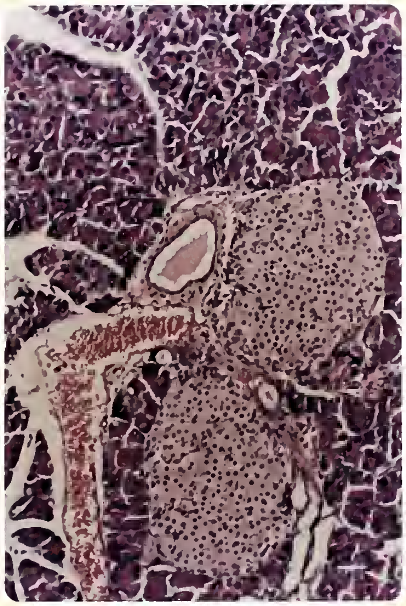

Fig. III normal mouse pancreas (x100)

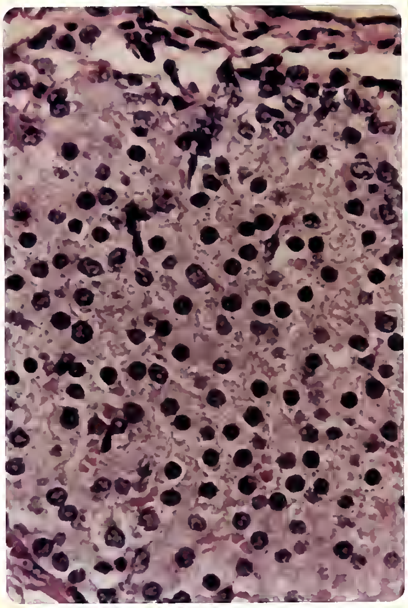

Fig. IV normal mouse pancreas (x400)

Of the 10 animals which received transplants to the anterior chamber, 5 proved to be unsuccessful (Nos. 5, 9, 47, 48, and 51) as evidenced by an inflammatory reaction and degeneration of the transplanted tissue. In effect then, these 5 mice received a sham operation and may be considered a second group of controls. They also exhibited significant glycosuria throughout the course of the experiment with the exception of mouse no. 5 which, shortly after the alloxan, voided 2 urine specimens free of glucose. At autopsy the pancreata of these mice showed changes similar to those seen in Fig. I.

Five of the transplants to the anterior chamber grew to fill this space and were considered to be successful grafts. Three of these mice each had received one embryonic dba pancreas (Nos. 6, 7, and 8) and 2 each were the recipients of 1 newborn dba pancreas (Nos. 49 and 50). From 2-6 weeks post

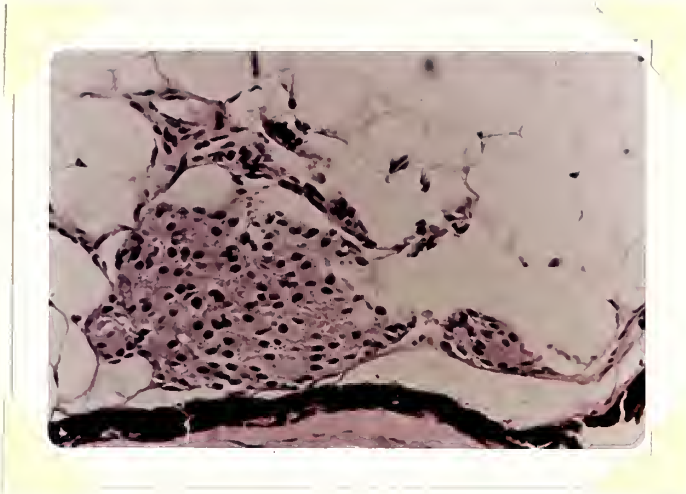

Fig. V from mouse no. 7
(x250)

transplant no. 7 had no demonstrable glycosuria. From the 6th through the 14th week he continued to spill 1-2+ sugar in the urine. After removal of the transplant by enucleation in the 15th week, the mouse voided urine containing greater than 0.7% (3-4+) glucose. Its enucleated eye was found to contain degenerating pancreatic tissue composed mostly of cystic spaces and fat. However, many islets were still present, and a section through one of these is shown in Fig. V. No acinar components were visualized in the sections examined. At autopsy 1 month after enucleation the pancreas of no. 7 exhibited changes similar to those in Fig. I, and in addition there was marked hyaline degeneration (Fig. VI and Fig. VII).

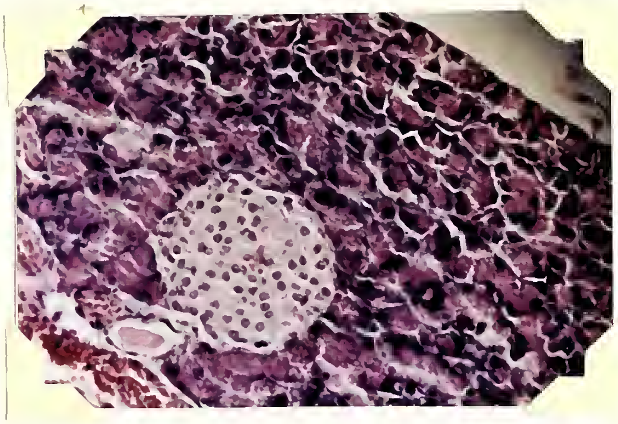

Fig. VI from mouse no. 7
(x100)

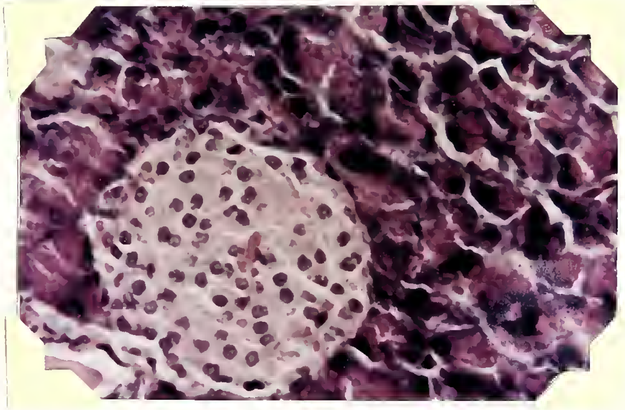

Fig. VII from mouse no. 7
(x250)

The pancreata of most of the remaining mice which were autopsied revealed degenerative changes in the islets resembling closely those of Fig. I or Fig. VII or a combination of both.

The other 4 mice with apparently successful transplants also had transient periods of sugar-free urine: nos. 50 and 49 in the 9th and 8th weeks respectively, no. 8 in the 4th week post transplant, and no. 6 one week after transplant. It is doubtful whether the urine free of glucose in the latter mouse is of any significance, since 1 week post transplant the graft had not yet attained its full size, and probably had not reached its full physiologic potential. Furthermore, the enucleated eye of no. 6 contained only a relatively small amount of islet tissue in comparison with that present in the eye of no. 7. The anterior chambers of mice nos. 49 and 50 contained more islet tissue than that present in no. 6 but substantially less than that recovered from no. 7.

Mice nos. 49, 50, and 6 showed no significant change in the degree of glycosuria after the transplants were removed. The pancreata obtained at autopsy from these mice were examined closely to ascertain whether the islets were less severely involved than those of no. 7, but it could not be established from the sections available. If this had proved to be the case, one might have conjectured that the diabetic state created in the former mice was of such a mild degree that their own pancreata were functioning well enough to prevent marked glycosuria, and that the insulin produced by the transplanted pancreatic tissue was insignificant in comparison to that secreted by their own normal islet cells.

Mouse no. 8 had a successful anterior chamber transplant and was not enucleated as were the 4 aforementioned mice. This mouse also had one period during which he was free of glycosuria and subsequent to which he continued to spill 1-2+.

Fifteen mice each received a transplant of 2 embryonic dba pancreata into the axillary subcutaneous space, and 5 animals each received 2 C_3H pancreata in the 17th day of gestation into the same site. At no time during the course of the experiment except immediately after transplant did any of the latter 5 mice void urine specimens that were free of glucose, and none of the 4 mice in this group who survived past 2 weeks post transplant developed any palpable axillary mass.

On the other hand, 9 of the 15 mice who had subcutaneous transplants of dba pancreas developed clinically visible and palpable masses in the axilla (Nos. 10, 11, 12, 13, 32, 33, 36, 39, and 40). One such tumor partially dissected away is shown in Fig. VIII.

Fig. VIII from mouse no. 33

All 9 of these mice had periods of no demonstrable glycosuria which occurred anywhere from 2-10 weeks after transplantation. The longest period of sustained negative glycosuria in the mice was seen in no. 11 during the 14th-54th days. The transplants were allowed to remain in situ in nos. 10 and 39. Both of these mice had urine free of glucose for a two week period and subsequently returned to the diabetic state with urines containing greater than 0.1% glucose but never greater than 0.6%. In the remaining 7 mice an attempt at removal of the palpable tumor was made. It proved difficult to completely remove the transplants because of a close resemblance of the pancreatic tissue to surrounding subcutaneous fat; however, as much as could be identified was excised. Fig. IX is taken from a section through the tumor of mouse no. 11 removed 14 weeks after transplant during the time the animal was clinically free of diabetes, and shows abundant amounts of islet tissue contained within cystic spaces adjacent to intestinal mucosa which apparently was part of the original graft. Fig. X is a closeup of the area outlined in ink and shows the somewhat bizzarely shaped islet cells.

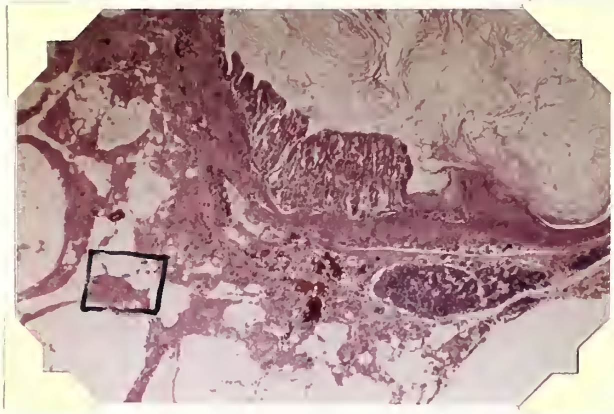

Fig. IX from mouse no. 11
(x100)

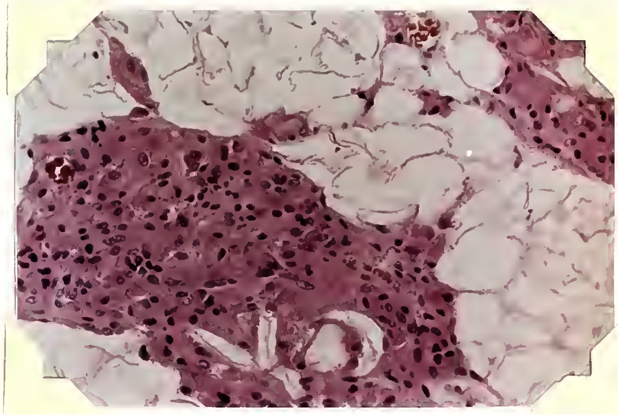

Fig. X from mouse no. 11
(x400)

After surgery, the 3 mice in Group I (Nos. 11, 12, and 13) developed a more severe glycosuria than they had ever exhibited previously. Mouse no. 23 died at surgery. The remaining 3 animals in Group II (Nos. 33, 36, and 40) did resume their former diabetic state after removal of the grafts, but glycosuria was never greater than 0.3% in any of them, and whether removal of the transplanted pancreatic tissue had any effect on the diabetes in these animals is equivocal. It should be emphasized, however, that the operator could in no way be certain that he had removed the transplanted tissue completely.

Of the remaining 6 mice which received subcutaneous dba transplants but developed no palpable subcutaneous masses, 2 (Nos. 35 and 41) each had a prolonged period post transplant during which their urine was free of glucose. Mice nos. 14, 34, and 38, however, showed no evidence that the grafts had become functional. The pancreata of all these mice at autopsy revealed the degenerative islet cell changes previously noted in the other animals, and in mouse no. 14 there was striking vacuolization and dropping out of cells seen in Fig. XI and Fig. XII. This animal had been the most severely diabetic of the entire group, losing several gms. of weight and consistently having 4+ glycosuria.

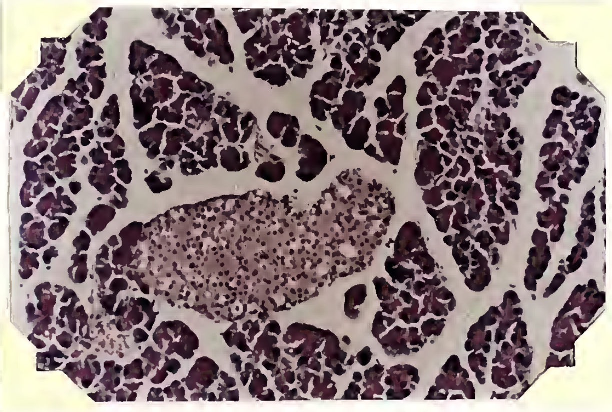

Fig. XI from mouse no. 14
(x100)

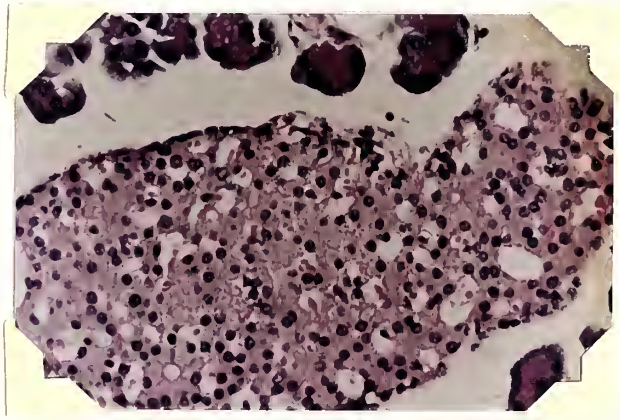

Fig. XII from mouse no. 14
(x400)

DISCUSSION AND SUMMARY

The data accumulated in the above experiment indicate that a mild diabetic state in mice can be controlled by transplants of embryonic pancreas. In 11 out of 15 diabetic hosts who received transplants of 2 embryonic pancreata into the subcutaneous space there was a period of remission during which time the diabetes seemed to be controlled. The results in the animals who were the recipients of grafts to the anterior chamber were less encouraging, and only one of several mice in this group showed any real evidence that the transplanted tissue was physiologically active. In 3 others the results were equivocal. Shortly after removal of the pancreatic transplants from the abovementioned 12 mice in which the tissue appeared to be functional, the animals became severely diabetic.

The failure of many of the grafts to take, especially those of embryonic C_3H pancreas might well be explained by the fact that the tissue used for the transplants was already in the last trimester of gestation, and Greene has pointed out that the property of heterotransplantability is possessed by embryonic tissue generally only during the first half of gestation[6]. The reason why the pancreatic acini did not persist in the grafted tissue, and why much of the transplanted islet tissue began to degenerate after it had survived 3-4 months in the hosts is not entirely clear. Whether pancreatic transplants could assume a permanent endocrinologic role in a diabetic host has yet to be determined.

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