Diabetologia, 1, 91–96 (1965)

Homografting of Fetal Rat Pancreas*

By

ALAIN E. GONET and ALBERT E. RENOLD

Institut de Biochimie Clinique, Université de Genève

Received July 5, 1965

Summary. Successful homografts of fetal rat pancreas into the testis of adult alloxan diabetic rats have been obtained in approximately 20% of a large series of experiments. Success was defined by graft survival over periods of 3 weeks or more, with evidence of multiplication of the transplanted endocrine cells. Histologic examination has demonstrated the existence of 3 morphological types of grafts 1. solid grafts; 2. scattered islet grafts; 3. pseudo-canalicular grafts. The endocrine cells were predominantly of the *B* type and typical β granulations could be demonstrated in all types with appropriate stains. Furthermore, acid ethanol extracts of the grafts exhibited considerable insulin-like activity on rat epididymal adipose tissue, activity which was completely suppressed by the addition of anti-insulin serum. The insulin content varied between 5 and 1200 units per gram and thus approached, in the solid grafts, the theoretical level for pure *B* cells. Although there was some suggestive evidence in favour of endocrine function of the graft within the host, the data are not as yet sufficient to establish this fact. Additional experiments will be needed and are in progress.

Résumé. Il est possible de faire des homogreffes de pancréas de rat foetal dans le testicule de rats adultes diabétiques, et d'obtenir dans les 20% des cas la survie et la multiplication des cellules endocrines implantées. L'étude histologique a montré trois types morphologiques de greffon: 1. les greffons solides, 2. en îlots disséminés, 3. de type pseudo-canaliculaire. Les colorations histologiques, telles l'aldéhyde-thionine mettent en évidence des granulations intracytoplasmiques semblables aux granulations β des cellules *B* de LANGERHANS. Le dosage biologique sur le tissu adipeux épididymaire du rat met en évidence une activité insulinique du greffon pouvant varier de 5 à 1200 UI d'insuline par gramme. Cette activité insulinique est entièrement supprimable par le sérum anti-insulinique. Les auteurs analysent les rapports qui existent entre la présence des granulations β et l'activité insulinique dans les homogreffes pancréatiques; mais ils ne peuvent pas encore répondre à la question capitale: le greffon est-il ou n'est-il pas fonctionnel. La réponse sera probablement donnée par une expérimentation similaire, actuellement en cours, mais effectuée cette fois, chez les rats consanguins, ce qui permet d'espérer un taux très élevé de reprises des isogreffes et des études statistiques suffisantes.

Zusammenfassung: Erfolgreiche intratestikuläre Homotransplantationen foetalen Rattenpankreasgewebes ließen sich in etwa 20% der Versuche bei Alloxan-diabetischen Ratten durchführen. Als Kriterium für die erfolgreiche Implantation galt das Überleben der Transplantate über drei Wochen oder länger mit nachweisbarer Vermehrung der innersekretorischen Zellen. Auf Grund des histologischen Bildes lassen sich die Transplantate in 3 Typen unterteilen: 1. kompakte, 2. inselförmige und 3. pseudo-canaliculäre. Die innersekretorischen Zellen sind bei allen drei Typen fast ausschließlich β-Zellen mit typischen β-Granula, die histochemisch charakteristisch reagieren. Außerdem ließ sich in salzsauren Äthanolextrakten der Transplantate am Rattenfettgewebe meßbare Insulin-ähnliche Aktivität nachweisen. Diese Insulin-ähnliche Aktivität verschwand bei Zusatz von Anti-Insulin Serum vollständig. Der Insulingehalt betrug zwischen 5 und 1200 Einheiten pro Gramm Transplantat und entsprach somit in einzelnen kompakten Transplantaten nahezu dem theoretischen Insulingehalt reiner β-Zellpräparate. Obschon bei den diabetischen Trägern der Transplantate Hinweise auf deren innersekretorische Aktivität beobachtet wurden, genügen die bisherigen Resultate nicht für eine beweisende Dokumentation. Zusätzliche Experimente sind im Gange.

In considering transplantation experiments of an endocrine tissue, major concern is due to two central questions: the survival of the graft and the persistence of endocrine function. We have previously reported[1,2] on studies devoted specifically to the survival of homografts of fetal pancreas in adult diabetic rats. The present report concerns the morphology of the grafts, their insulin content, and their endocrine function.

Materials and Methods

The recipient animals were male albino Wistar rats bred in our own colony during the last two years. Alloxan diabetes was induced by intravenous injection of 40 mg per kg after a 14 hour fast. The diabetic state was partially controlled through the injection, twice daily, of doses of lente insulin sufficient to permit growth and to decrease glycosuria according to criteria which have been previously reported[2]. The doses needed were of the order of 8 to 40 units per kilogram per day. From the time of alloxanization the animals were kept in individual metabolic cages in a room at constant temperature and with controlled humidity. The pancreatic grafts were obtained from fetuses as near as possible to term, delivered by cesarean section. The caudal portion of the fetal pancreas was rapidly removed and minced with pointed scissors in a large drop of He-La Eagle's medium for the culture of mammalian tissues[3] obtained from Difco Laboratories, Detroit 1, Mich. USA.

* Supported by the Fonds National Suisse de la Recherche Scientifique, grant No. 3244 and by a grant in aid from Diabetes Foundation, Incorporated, Boston, Massachusetts.

The fragments were grafted into the testis of the recipient rats, either by placing them under the albuginea with a fine trocard or by injecting them into the testicular parenchyma with a tuberculin-type syringe and needle. From then on, according to the evolution of the diabetic state in each animal, the insulin injections were progressively decreased and, in certain instances, stopped. The transplantation technique has been described previously in more detail[1,2]. In the experiments reported here, transplantation was into one testis only in any one recipient animal.

As a rule, the transplants were examined four weeks after grafting. The testis carrying the transplant was removed through a hemicastration procedure and immediately frozen and cut with the microtome. On cut section the graft is easily seen because of its brownish colour. When it appeared on section, a slice was obtained for histology, while the remainder of the graft was carefully dissected from the seminiferous tubules, a dissection which is quite easy in the frozen state. The portion of the graft so obtained (one half or more of the total graft) was then weighed on a METTLER model M 5 microbalance and served for the assay of its insulin content, utilizing the adipose tissue biossay procedure developed in this laboratory[4]. The biossay was applied either to dilutions of the straight homogenate, or to dilutions of an acid ethanol extract of the homogenate[5]. The histologic techniques used included the following stains suitable for the differentiation of the endocrine granules: aldehyde-fuchsin according to WILSON's modification of the GOMORI technique[6], Victoria blue according to IVIC[7] and, principally, aldehyde-thionin according to PAGET[8], slightly modified in this laboratory for rat pancreas, (see the addendum, below).

After hemicastration and removal of the grafts, the animals continued in the metabolic cages and their glucosuria measured during 10 days.

Results

In all instances there was rapid degeneration of the exocrine portion of the pancreas which, as a rule, disappeared completely. In approximately 20% of the pancreatic homografts[2] proliferation of the endocrine portion occurred. This does not mean that 20% of fetal pancreatic homografts are tolerated, since in each instance more than one small pancreatic fragment is grafted, with corresponding increase in the chances of acceptance and with an increase in the surface available for metabolic exchanges with the tissue fluids. The fragments so grafted contained, in unpredictable fashion, one or more islets of LANGERHANS, portions of one, or none. *B* cells occurred in all grafts containing surviving islet tissue. It would seem likely at this time that the random aspect of the transplantation is at the basis of the three types of morphology seen upon histologic examination of the grafts. Approximately equal numbers of grafts presenting each of the three histologic types have been observed.

Solid grafts: these grafts, illustrated by those shown in Fig. 1 and 2, are likely to derive from a single fetal islet of Langerhans. Almost all the cells seen present morphological and histochemical characteristics usually associated with the B cells of the pancreas. As a rule, however, they are larger, with a mean diameter of 15.5 microns. They are disposed as cellular strands surrounded by a very abundant network of capillaries. These grafts are the largest and have reached 4 mm in diameter in the most favourable cases. We have never seen *A* cells in these transplants.

Islet-grafts: these grafts may well be derived from more than one fragment of fetal pancreas and appear as several islets distributed through the interstitial tissue of the testis. Their morphological and staining characteristics resemble in all instances those of the *B* cells of the islets of LANGERHANS (Fig. 3) although, again, the mean cell-size appears to be larger than that seen in the fetal or adult rat pancreas. *A* cells are rarely seen and have never been identified as such with certainty.

Pseudo-canalicular grafts: here the endocrine cells often surround empty spaces and appear to form canalicular or pseudo-canalicular structures (Fig. 4). This type of graft may well derive from small pancreatic ducts, although this is but a conjecture as of the present. The cells again exhibit the staining characteristics of *B* cells, but their morphological characteristics are somewhat different. They are more oblong or cylindrical and their nuclei are more often oval than spherical. Furthermore, although the β granules are evenly distributed through the cytoplasm, their number is less than in the cells of the other two types of grafts.

Biossays for insulin-like activity: the material for biossay was repeatedly checked histologically and was found to consist of graft tissue only, without host tissue, with exception of the formed blood elements contained in the blood vessels and capillaries. Only the grafts exhibiting a satisfactory macroscopic appearance were assayed. The values which have been obtained are shown in the Table. For comparison, it should be recalled that the insulin content of pancreas from newborn or adult rats is of the order of 1.5 to 5 units per g

Table. *Insulin-like activity (ILA) of acid ethanol extracts of 7 grafts. In each instance the activity was totally suppressed by the addition of anti-insulin serum obtained against porcine insulin in guinea-pigs*

Graft No.	ILA of Grafts Units/gm	Days after grafting	index of precision (λ) of bioassay
1	1200	16	0.09
2	5.8	28	0.07
3	58	28	0.16
4	20	31	0.25
5	5	29	0.15
6	400	29	0.15
7	3.2	29	0.10

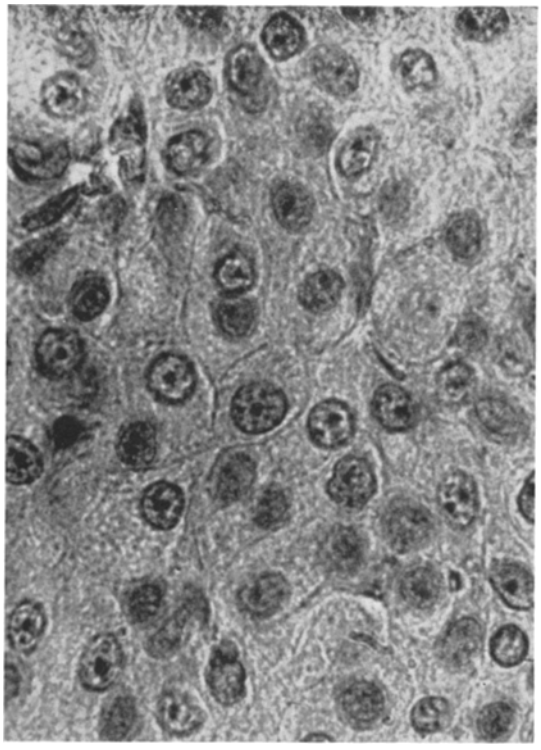

Fig. 1. Solid graft. Strands of cells exhibiting the morphological and staining characteristics of B cells of the islets of Langerhans. Most of the clearly visible fine granulations distributed throughout the cytoplasm appear blue in this aldehyde-thionin stained section. Enlarged 700 x

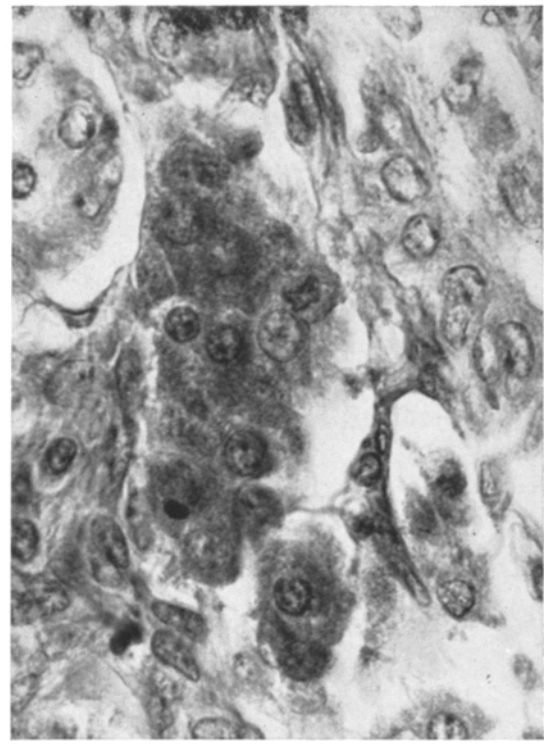

Fig. 3. Islet graft. An isolated islet consisting exclusively of B cells is seen here surrounded by interstitial cells of the testis. The granulations are dark blue in this aldehyde-thionin stained section. 700 x

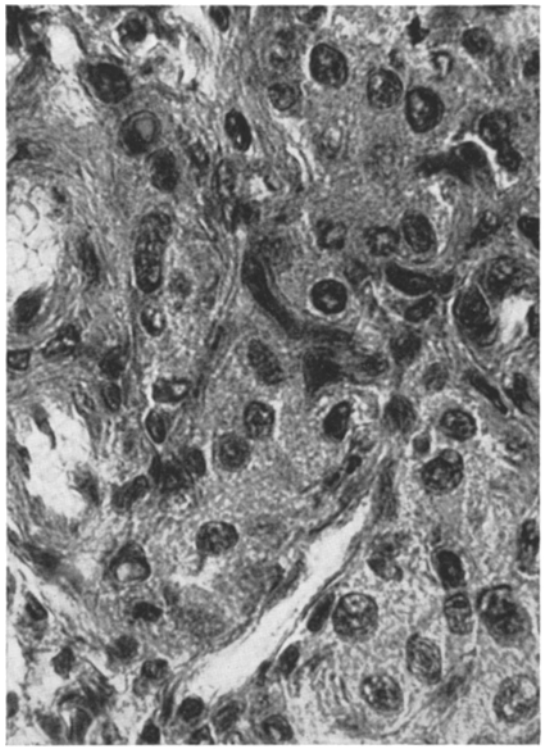

Fig. 2. Solid graft. The strands of endocrine cells are surrounded by an abundant net of capillaries. Again the β granulations (blue in this aldehyde-thionin stained section) are clearly visible. A few scattered fibrocytes can be seen. 700 x

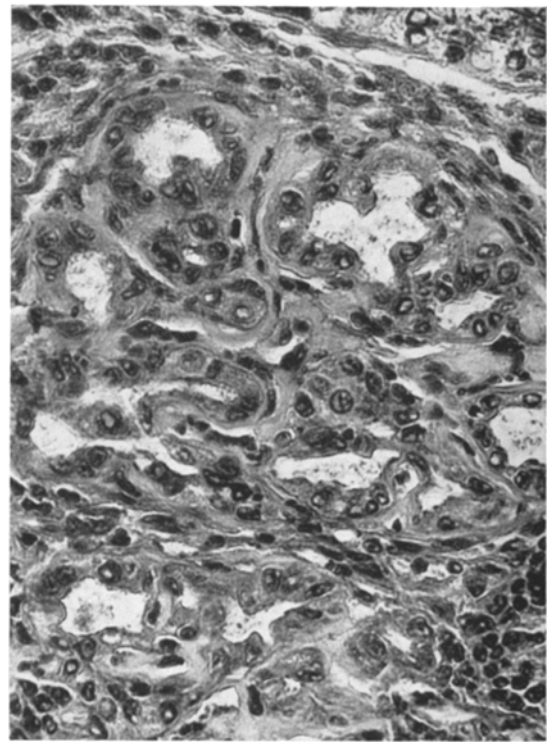

Fig. 4. Pseudo-canalicular graft. This canalicular appearance has been given the name of nesidioblastosis in human pathology. The cells which surround the empty spaces are endocrine in appearance and contain granulations which are blue in aldehyde-thionin stained sections. Their density is less than that seen in the solid or islet-type grafts, but their identification as β granules is nonetheless unquestioned. Between these endocrine pseudo-canalicular formations there are numerous fibrocytes. 400 x

wet weight[9,10], while the insulin content of isolated islets of LANGERHANS is of the order of 150 to 500 units per g[11] and that of isolated *B* cells of the order of 500 to 1200 units per g[12]. Fetal rat pancreas used for the transplants contained only between 0.25 and 0.35 units per g.

The insulin-like activity found in the seven grafts which have been analyzed varied rather widely (Table 1), from 5 to 1200 units per g. It is of interest that the activity so extracted exhibited, upon dilution, a dose-response curve strictly parallel to that of crystalline porcine insulin and that, in each instance, it was completely suppressed by anti-insulin serum obtained in guinea-pigs immunized with porcine insulin. It is of interest that the grafts with the greatest insulin content were of the "solid" type. Thus graft number 1 in the table, with 1200 units of insulin-like activity per gram, approaches the theoretical insulin content of pure *B* cells. This graft is that shown in Fig. 2 and thus appeared, histologically also, to consist exclusively of *B* cells with a larger average diameter (15.5 μ) than that of *B* cells in adult islet of LANGERHANS of normal rat. The diameter of the latter average 9 microns[13]. Also, the β granulations observed in this graft were particularly dense. Since the graft is richly vascularized, this high content of insulin-like activity suppressible with anti-serum and thus very likely insulin, is particularly striking.

Graft number 3, containing 58 units of insulin-like activity per gram exhibited an islet-type of distribution of the endocrine cells (Fig. 3), while grafts such as numbers 2, 5 and 7, which contained only small amounts of insulin-like activity, appeared to be still well vascularized but exhibited early fibrosis. They did, however, still present a few typical, isolated *B* cells.

Discussion

It would seem reasonable, therefore, to conclude that the technique which we have described is one suitable for obtaining endocrine pancreatic grafts which contain cells which exhibit morphological and staining characteristics of insulin-producing *B* cells, a conclusion which is greatly strengthened by the demonstration in all of these grafts of a material with biological insulin-like activity, an activity which is furthermore suppressed by the presence of an anti-insulin serum. Occasionally, grafts are obtained which appear to consist almost exclusively of *B* cells, as illustrated both by their histological appearance and by the extremely high content of insulin-like activity approaching that of pure *B* cells. Comparison of fetal pancreas with the appearance of the graft, 4 weeks after transplantation, clearly establishes that there has been growth of the grafts after transplantation, and not just survival of transplanted cells.

The differences which have been observed in the histological appearance of the grafts, described above as solid grafts, islet-grafts, and pseudo-canalicular grafts, have also been described in patients with hypoglycemia associated with pancreatic tumors. PATEL and collaborators[14] have emphasized three types of appearance: firstly that of a giant islet of LANGERHANS with cuboid or cylindrical cells arranged as well-vascularized strands, with well-defined cytoplasmic granules. Secondly, smaller aggregates of endocrine cells scattered through the gland, or through areas of the tumor, consisting of epithelial cells which are less well defined. PORTER and FRANTZ[15] have suggested that this type of tumor is primarily seen in patients with multiple tumors, either pancreatic or polyglandular. Thirdly, in human tumors as well as the grafts which we have studied, the cells may be disposed as rosettes or tubules, quite similar to the appearance of the pseudo-canalicular grafts. These cells appear to be functional; they border on capillaries and typical endocrine granulations have been demonstrated both with light and with the electron microscope[16].

The intratesticular transplantation technique described here is not the only type of endocrine pancreatic homograft for which positive histological results can be claimed. Several laboratories have utilized successfully the hamster cheek pouch for pancreatic auto-, iso-, and less frequently, homo- grafts[17,18,19,20]; others the anterior chamber of the eye[21,22,23,24]. In general, the size of the grafts so obtained has been very much smaller, and the endocrine cells more scattered than in the intratesticular grafts. The presence of insulin activity in hamster cheek pouch grafts has been reported in abstract form by SAK and collaborators[25].

What is the functional activity of the intratesticular pancreatic hormgrafts? At present no decisive answer can be given to this question. Many animals exhibit a tendency to hypoglycemia during the first 24 to 48 hours following grafting. However, since the insulin activity contained in the fetal grafts is negligible, this observation is most likely related to postoperative alterations in the pattern of food intake. In most instances, this early tendency to hypoglycemia disappears despite continued insulin treatment, and in most of these instances only inflammatory tissue or scarring are found upon histological examination. A more prolonged decrease in glycosuria and in the levels of blood glucose is seen in approximately twenty percent of the grafted rats, and it is in this group of animals that we have found the largest and best preserved grafts containing measurable insulin activity suppressible with anti-insulin serum, as well as excellent histological evidence of β granules. A considerably greater body of evidence will have to be gathered, however, before it will be possible to correlate in more detail the type of graft appearance and the evidence for its physiological activity. Although some increase in glycosuria was observed in all of the grafted animals after graft removal through the hemicastration procedure, it has again proved difficult to endow these results with significance, because of the

difficulty of interpreting the contribution of the surgical procedure itself. Also, the not infrequent regeneration of the islets of LANGERHANS of the alloxanized host[26] further complicates the situation throughout. Accordingly, the only appropriate present conclusion must be that the function of the graft tissue is fully substantiated by the histological appearance of the *B* cells and by their content in insulin activity which is both biologically and immunologically similar to insulin. However, it is not possible to state whether secretion in the host occurs to an extent sufficient to modify the diabetic state.

It is hoped that an answer to this last question will be obtained in a present series of experiments dealing with isografts in inbred Wistar rats exhibiting crossed tolerance to skin grafts. With a larger and more predictable number of "takes" an answer to many of the questions which remain open as the result of the present study may become possible.

Acknowledgments

We gratefully acknowledge the expert technical assistance of Miss JANINE MOUGIN and Miss JOSETTE C. LEUBA.

The Aldehyde-thionin staining method was modified and adapted to our procedure by Miss MOUGIN.

The insulin used in these studies was kindly supplied through Dr. ERNST GMACHL of ELI LILLY and Company.

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Addendum: Modification of the aldehyde-thionin stain for endocrine pancreatic granules:

Solutions:

I. Thionin (MERCK, 1421) 500 mg
Ethanol 70% 91.5 ml
Paraldehyde (Merck 7165) 7.5 ml
Concentrated HCl 1.0 ml
Ready to use after two days.
May be kept a few weeks if well stoppered.

II. Azocarmine G (MERCK, 1593) 100 mg
Distilled H_2O 100 ml
Bring to a boil, then cool to 60° C and add glacial acetic acid. 1 ml
Prepare immediately before use.

III. Potassium permanganate	300	mg
Distilled H_2O	50	ml
0.6% H_2SO_4	50	ml

Prepare immediately before use.

Fixation: It is recommended to use BOUIN's solution, dehydration as usual and embedding in paraffin, with sections of 5 μ thickness.
Technique: Post-fixation in Bouin's solution at 60°C for 15 minutes. Wash in tap water 1—2 hours to eliminate picric acid.
Oxidation in Solution III for 7 minutes. Wash in tap water. Bleach sections in 3% sodium metabisulfite and wash 10 minutes in tap water, then rinse in 70% ethanol.
Stain with aldehyde thionin (Solution I) at least 30 minutes in closed vessel; then wash in running tap water.
Stain the nuclei in BOEHMER's hematoxylin, 5 minutes, and rinse in running tap water.
Stain with azocarmine G (Solution II) one minute at 60°C.
Rinse two or three times in water at 40°—50°C, then in tap water.
Dehydrate in ethanol-xylol according to standard techniques and mount in balsam.

Dr. A. E. GONET
Institut de Biochimie Clinique
Sentier de la Roseraie
Genève/Suisse