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12. The drugs used, concentrations, and *p*H values were: *dl*-norepinephrine hydrochloride (Aldrich), 0.5*M*, *p*H 4.0; acetylcholine chloride (Merck), 0.5*M*, *p*H 4.5; guanosine 3′,5′-monophosphate (Sigma), 0.5*M*, *p*H 6.0; N^6,$O^{2'}$-dibutyryl adenosine 3′,5′-monophosphate (Sigma), 0.5*M*, *p*H 8.0; and N^6-monobutyryl adenosine 3′,5′-monophosphate (Sigma), 0.5*M*, *p*H 5.5.
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Beta Cell Culture on Synthetic Capillaries: An Artificial Endocrine Pancreas

Abstract. *Beta cells from neonatal rats were cultured on bundles of artificial capillaries perfused with tissue culture medium. Cells continued to release insulin and remained responsive to changes in glucose concentration. The quantity of insulin released was similar to that of conventional flask cultures.*

The application of tissue culture techniques to the study of pancreatic beta cells offers a number of distinct advantages. Monolayers that have been prepared from pancreases of newborn rats can be maintained for periods varying from days to months (*1*). They have been successfully employed to study beta cell growth and replication, insulin biosynthesis and release, and beta cell morphology by both light and electron microscopy (*1*, *2*). In addition, small numbers of alloxan diabetic animals have been treated by implanation of these cultured cells (*3*).

Until recently these monolayers were cultivated on the surfaces of plastic or glass petri dishes or flasks. In 1972 Knazek *et al.* (*4*) reported culturing cells from established lines on bundles of artificial capillaries formed from plastic. In the present studies these methods were adapted to culturing normal diploid pancreatic beta cells.

The overall rationale was: (i) to examine possible means for improving the growth of pancreatic beta cells compared to conventionally utilized petri dish or flask systems; (ii) to develop a convenient method for studying short-term insulin secretory dynamics by using preparations maintained for days, weeks, or months under varying controlled conditions; and (iii) to develop a prototype for an artificial endocrine pancreas, since cells cultivated in this way are protected by a membrane barrier, and thus are not vulnerable to immune rejection (*5*).

One hundred fibers (Amicon XM-50) were sealed into the ends of a small glass jacket. The lumen of each fiber was 190 μm in diameter and the wall thickness 75 μm. It was permeable to substances with molecular weight below 50,000. The total capillary surface available for cell attachment was 75 cm^2. Plastic flasks with surface area 75 cm^2 were used for control cultures. Cells were prepared from the pancreases of newborn rats as previously described (*1*, *2*) and seeded into either capillary units or flasks (*6*). Capillary units were perfused with 100 ml of tissue culture medium recirculated at a rate of 2 to 3 ml per minute. Silastic tubing connecting the capillary units

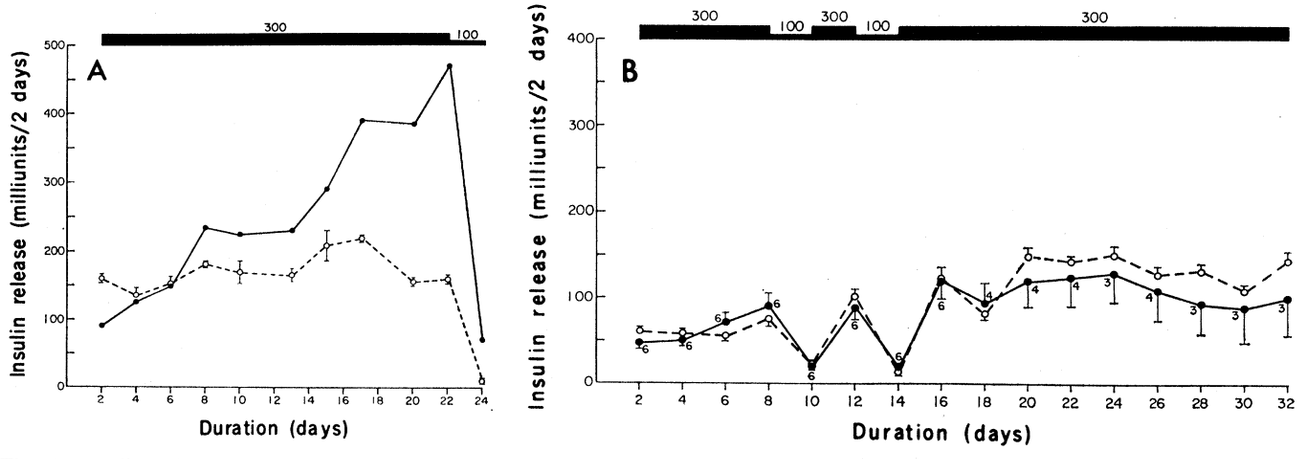

Fig. 1. Insulin release from representative capillary units versus insulin release from conventional flask cultures. The glucose concentration (300 or 100 mg per 100 ml) was varied as indicated by the labels on the heavy bars. (A) Solid line: one capillary unit; dotted line: mean of three flasks, plus or minus the standard error of the mean. (B) Solid line: mean of capillary units (*N* as indicated) ± S.E.M.; dotted line: mean of eight flasks ± S.E.M.

with the reservoirs of medium served as gas exchangers (*7*).

The immediate goals of the experiments were: (i) to determine whether beta cells cultured on these hollow fibers continued to secrete insulin; (ii) to ascertain whether the quantity of insulin released compared favorably with that from flasks seeded with equal numbers of cells from the same suspension; (iii) to determine whether insulin release would respond appropriately to alterations in the glucose concentration of the perfusate; and (iv) to examine the light microscopic morphology of beta cells cultured in the system.

In one series of experiments insulin release from a capillary unit maintained at 100 mg of glucose per 100 ml was studied for 24 days. In general it was somewhat greater than that from control flasks prepared from the same cell suspension. When the glucose concentration was raised from 100 to 300 mg per 100 ml during the last 2 days of the experiment (days 22 to 24) there was a fourfold increase in the quantity of insulin released from the capillary unit. In the reverse experiment (Fig. 1A) cultures were maintained at a glucose concentration of 300 mg per 100 ml, which was lowered to 100 mg per 100 ml during the last 2 days. In this case, the quantity of insulin released from the capillary unit was considerably greater than that from the flasks. Lowering the glucose concentration in the perfusate caused a large reduction in insulin release.

In addition, several units were put through successive cycles of high and low glucose in the perfusate (Fig. 1B). Again, it was readily apparent that insulin release responded to changes in glucose concentration.

To date we have successfully studied a total of 12 capillary units. The longest any were maintained was 5 to 6 weeks, at which time the experiment was terminated for morphologic evaluation.

In order to increase the quantity of insulin released from capillary units, a technique was developed for concentrating beta cells from the initial cell suspension by gradient centrifugation, at a yield of roughly 30 percent (*8*). Each of two units was seeded with a suspension of "concentrated" beta cells from 180 pancreases. After 8 to 10 days of incubation, insulin release had risen to the range of one unit per 2-day interval. One of the units was studied for a total of 40 days, and insulin release remained at approximately this same level (Fig. 2).

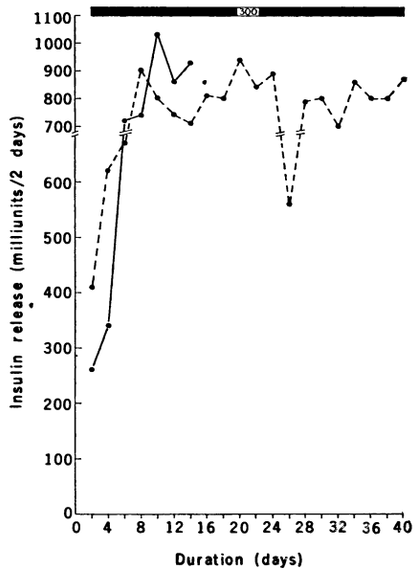

Fig. 2. Insulin release from capillary units seeded with concentrated preparations of beta cells. Solid line: capillary unit No. 1; dotted line: capillary unit No. 2. The glucose concentration was 300 mg/100 ml.

Examination of aldehyde-fuchsin stained sections of representative capillary bundles confirmed the presence of clusters of viable beta cells with an abundance of aldehyde-fuchsin positive granules (Fig. 3). In addition to rounded clusters, small groups of these cells were situated around the periphery of the fibers and occasionally appeared to fill completely the spaces between closely adjacent fibers.

These data indicate that beta cells cultured on artificial capillaries continue to synthesize, store, and release insulin. Moreover, insulin release can be modulated by alterations in the glucose concentrations of the perfusate. The quantity of insulin released was generally similar to that of flasks seeded with equal numbers of cells. In two cases, however, one of which is shown in Fig. 1A, insulin release appeared greater. It is presently unclear whether this was due solely to chance variation. It is noteworthy, however, that gonadotrophin release by human choriocarcinoma cells is considerably enhanced by culture on artificial capillaries as compared to flasks (*4, 7*). This is due to both increased cell replication and increased hormone release per cell. In these experiments (*4, 7*), silicone polycarbonate capillaries were mixed with the Amicon XM-50 fibers to facilitate oxygen transport from the perfusate to the extracapillary medium. The importance of this maneuver in enhancing insulin production and beta cell replication remains to be investigated. In addition, at times, significant numbers of pancreatic cells adhere to the inner surface of the glass jacket rather than the capillary fibers. This may also be of significance when comparing the performance of capillary units and flask cultures.

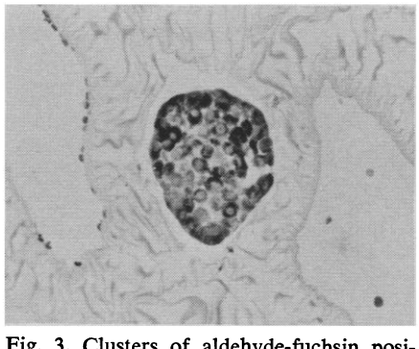

Fig. 3. Clusters of aldehyde-fuchsin positive beta cells on the surfaces of capillary fibers. Cells were fixed in situ with 10 percent formalin and embedded in 2 percent agarose in normal saline (*4, 7*). Units were opened, the cylinder of agarose was removed, and portions were embedded, sectioned, and stained by conventional methods (*10*). Aldehyde-fuchsin stained (× 230).

In view of these findings, it is not unlikely that these capillary units could be adapted to studying short-term insulin secretory dynamics by modifying the perfusion circuit. Such studies could be performed, for example, after an initial longer period of perfusion with media of varying composition. In addition, these units appear to merit further consideration as a prototype for the development of an artificial implantable living endocrine pancreas.

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Conjugal Transfer of a Chromosomal Gene Determining Production of Enterotoxin in Vibrio cholerae

Abstract. *Matings between strains of* Vibrio cholerae *differing in toxinogenicity, nutritional requirements, and antibiotic susceptibilities were performed in order to determine the location of the gene* tox *that controls production of cholera enterotoxin. Segregation analysis shows that* tox *is linked to a gene required for histidine biosynthesis. Our data indicate that the* tox *gene is located on the bacterial chromosome and not on a plasmid in the strains of* V. cholerae *studied.*

Although it is clear that a protein enterotoxin (known as choleragen) is responsible for the diarrhea of cholera, little is known regarding the regulation of toxinogenesis in *Vibrio cholerae* (*1*). In some strains of *Escherichia coli* the gene *ent* that controls production of enterotoxin has been shown to be located on a plasmid (*2*). Because the heat-labile protein enterotoxins of *V. cholerae* and of *E. coli* are immunologically cross-reactive and have similar modes of action (*3*), it is of interest to determine whether or not the production of enterotoxin by *V. cholerae* is also regulated by plasmids. Although a mating system resembling the F-mediated system of *E. coli* has been described in *V. cholerae* (*4*), conjugal mating promoted by the vibrio sex factor P has not previously been used to study the genetics of toxinogenesis in *V. cholerae*. In the present study, a gene controlling the synthesis of cholera enterotoxin has been transferred by conjugation from a toxinogenic (*tox*+) strain to a nontoxinogenic (*tox*−) strain of *V. cholerae*. In addition, segregation analysis indicates that the *tox* gene is linked to a chromosomal gene that controls the biosynthesis of histidine.

Several strains of *V. cholerae* were used in this study. *Vibrio cholerae* 569B Inaba, the widely used and highly toxinogenic strain (*1*), was found to be a genetic recipient (P−). It was converted to a genetic donor (P+) by methods previously described by Parker and Romig (*5*). *Vibrio cholerae* RV-31 (P−) and RV-102 (P+) are strains of Ogawa serotype and were provided by W. R. Romig (Department of Bacteriology, University of California, Los Angeles). Strain 569B is prototrophic as well as toxinogenic (*1*), while strains RV-31 and RV-102 have auxotrophic and antibiotic resistance markers characterized in earlier studies (*6*). All pertinent characteristics of strains 569B and RV-31 are given in Table 1, and strain RV-102 is described below. Strains RV-31 and RV-102 were tested for production of enterotoxin both in vivo and in vitro by methods described previously (*7*). Strains RV-31 and RV-102 produced no detectable toxin in vitro and did not elicit secretory responses in ligated ileal loops of adult rabbits in multiple tests.

The conditions for mating experiments found to be optimal by Parker *et al.* (*8*) were used. Briefly, cultures of a prototrophic, *tox*+ donor and a multiply auxotrophic, *tox*− recipient were mixed to give a 1 : 10 ratio of donor to recipient cells. The mating mixtures were incubated at 37°C for 2 hours with gentle agitation. Washed and concentrated samples of the mating mixtures were mixed with molten soft agar and plated on appropriately supplemented minimal media in order to select for individual nutritional markers from the donor. The donor was counterselected by its susceptibility to streptomycin. The frequencies of cotransfer of all unselected markers were then determined in each class of recombinants. In order to determine requirements for amino acids, recombinants were transferred to separate plates lack-

Table 1. Segregation of toxinogenicity during recombination in *Vibrio cholerae*. Donor: 569B (P+) *arg*+ *his*+ *ilv*+ *str*^{s} *tox*+; recipient: RV-31 (P−) *arg*− *his*− *ilv*− *str*^{r} *tox*−. ND = not done.

Selected marker from donor*	Experiment number	Recombination frequency†	Number of recombinants tested	Number of selected recombinants with unselected marker from donor			
				tox+	*his*+	*ilv*+	*arg*+
his+	1	2.5×10^{-7}	23	4		4	20
	2	1.1×10^{-6}	100	7		0	0
	3	1.0×10^{-6}	120	3		0	0
	4	1.2×10^{-6}	322	11		ND	ND
	5–10	3.6×10^{-6}	900	21		ND	ND
ilv+	1	5.0×10^{-6}	100	1	2		3
	2	8.5×10^{-7}	140	0	0		1
	3	5.2×10^{-7}	120	0	0		3
	4	1.1×10^{-6}	305	1	ND		ND
arg+	1	4.0×10^{-6}	100	0	0	3	
	2	8.5×10^{-7}	100	0	1	2	
	3	9.0×10^{-7}	120	0	0	0	
	4	7.1×10^{-7}	283	0	ND	ND	

* Selected marker from recipient was *str*^{r}. † Expressed as the number of recombinant colonies detected per input donor cell.