Factors Influencing Isolation of Functional Pancreatic Rat Islets

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Abstract: Yields and function of isolated islets vary considerably in spite of the introduction of new or improved methods for isolation. In most studies, these variations have been attributed to inadequacies of the applied collagenase preparations. However, when we retrospectively analyzed our rat islet isolations, we found large variations in yield and function in spite of application of identical collagenase sources. Therefore, in the present study, we determined the effect of rat donor strain, the source of inhibition of proteolytic activity (by bovine serum albumin), and the culture conditions on yield and function. AO rats showed a twofold higher islet yield than Wistar and Lewis rats. However, a higher yield was not associated with a higher response on glucose load since this response was more pronounced with Lewis islets than with Wistar and AO islets. Rats with a higher weight donate more islets but have a lower insulin secretory capacity. Islet yield and function also vary with application of different sources of bovine serum albumin during digestion. Moreover, the culture conditions influence the functional survival of isolated rat islets. CMRL1066 preserves the insulin secretory capacity of rat islets better than RPMI1640. Finally, the number of islets surviving the culture is higher when 4 instead of 12 and 24 islets were applied per square centimeter. Our observations indicate that strain and weight of the rat donor, the source of bovine serum albumin, and the culture conditions of islets are pertinent factors in efficacious isolation of islets.

Key Words: islets, transplantation, bovine serum albumin, rat, strain, collagenase

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During the past decades, a large number of procedures for isolation of islets from the pancreas have been described.[1–3] Unfortunately, most of the procedures are characterized by insufficient reproducibility and inadequate efficacy.[3] This inadequate efficacy is illustrated by the generally accepted concept that multiple donors are required for successful clinical and experimental transplantation of pancreatic islet.[3–6] More insight into the factors determining the efficacy of islet isolation will predictably improve both the yield and functional survival of the tissue.

Islet isolation requires dissociation of the pancreatic tissue without affecting the structural and functional integrity of the islets. Pancreatic tissue is usually digested with commercial *Clostridium histolyticum* collagenase, a mixture of at least 6 collagenotic enzymes and various other enzymes with proteolytic activity.[7,8] In previous studies, we have shown that the composition of the collagenase,[7,9] especially the content of neutral protease, is a pertinent factor in the success of the isolation process. However, when we applied this collagenase on the rat pancreas, we were not able to isolate more than approximately 40% of the islets present in the naive pancreas, and the yield varied between 400 and 1200 islets per pancreas.[3,10,11] Obviously, other pertinent factors than the collagenase composition also influence the efficiency of islet isolation.

The present study was undertaken to identify other factors influencing the efficacy of the isolation procedure of pancreatic islets in the rat. Identification of such factors is mandatory for standardization of the islet isolation procedure to reduce variations and increase efficacy in function and yield of isolated islets. We have investigated the influence of the strain and weight of the applied rat donor, the effect of different source of bovine serum albumin (BSA) to suppress proteolytic activity during digestion, and the culture conditions of islets since we have repeatedly observed differences in yield and function of islets when one of these factors was changed.

METHODS

Design of the Study

Effect of Islet Donor Characteristics

Albino Oxford (AO), Lewis, and Wistar rats served as islet source. AO rats weighed 240–260, 300–320, and 350–370 g. All other strains weighed 300–320 g. Islets were isolated with 1 type and batch number of collagenase. We used the perifusion method with collagenase on pancreas fragments instead of intraductal injection since we found in a previous study a higher yield with this method.[12]

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Effect of BSA Source

Three sources of BSA were used. Two sources were obtained from Sigma but differed in their batch number while the third was obtained from Serva. Only fraction V BSA was used. The number and diameters of islets per isolation were assessed during hand picking. Islet function was determined by a static glucose challenge test immediately after isolation.

Effect of Culture Conditions

Islet function and number of surviving islets were determined after culture for 2, 5, and 7 days in CMRL1066 or RPMI1640 containing 5.5, 8.3, or 11.1 mmol/L glucose. The same assessments were used when islets were cultured at a density of 4, 12, or 24 islets per square centimeter.

Tested Materials

Crude *C. Histolyticum* collagenases are composed of 2 types of collagenase enzymes and several proteases.[10] On the basis of more than 2000 islet isolations with more than 50 different lot numbers of collagenases, we have defined activity limits for furylacryloyl-Leu-Gly-Pro-Ala (FALGPA) hydrolysis, collagen digestion, neutral protease activities, trypsin activity, and clostripain (Table 1) to allow adequate isolation of rat islets. Commercial collagenases with other activity were associated with either dramatic low yields or nonfunctional islets. The collagenase (lot numbers 104H68681: FALGPA, 3.1; collagen digestion, 1370; neutral protease, 62; trypsin, <0.05; clostrapain, 1.0) used in the present study was within the defined activity limits.

BSA was obtained from Sigma (lot numbers 51K1339 and 26H1012) and Serva (lot number 14177). The BSA was always of fraction V since we found that only this fraction was associated with adequate trypsin inhibition[3,11] and high islet yields.

Islet Isolation

Male inbred AO (AO/G), Wistar/G, and Lewis rats were obtained from Harlan or the Central Animal Laboratory of Groningen. All donors weighed 300–320 g or as otherwise specified.

Islets were isolated as follows.[3] Islet isolation was performed in Krebs-Ringer-HEPES (KRH) buffered with 25 mmol/L HEPES containing 10% (wt/vol) BSA. After ductal distention of the pancreas with KRH containing 10% BSA, the organ (929 ± 65 mg) was chopped into 1-mm^2 pieces. Next the tissue fragments were subjected to 3 successive washing steps with KRH containing 10% (wt/vol) BSA to remove proteases that might have leaked out of the exocrine part of the pancreas. Subsequently, the chopped pancreas was brought into a 25-mL Erlenmeyer flask and incubated at 37°C with 1.0 mg/mL of collagenase with KRH containing 10% BSA. The total volume of tissue and buffer was always 13 mL. After 10 minutes, the tissue fragments were placed in a 50-mL tube with 30 mL KRH containing 10% BSA at 4°C. The digested tissue was washed and sedimented 2 times to remove collagenase and leaked exocrine-derived proteases. Next, the tissue fragments were placed again in a 25-mL Erlenmeyer flask and incubated at 37°C with 0.7 mg/mL of collagenase with KRH containing 10% BSA in a total volume of 13 mL. After 8 minutes, the tissue fragments were placed in a 50-mL tube with 30 mL KRH containing 10% BSA at 4°C. Finally, the digest was allowed to sediment and was washed twice with RPMI containing 1% BSA. This 2-step collagenase isolation procedure allows a rather fast digestion in the first step and a more extended and precise digestion in the second step to remove small amounts of exocrine tissue around islets in the absence of dramatic loss of clean islets.

Islets were separated from exocrine tissue by hand picking.[13] For quantification of the endocrine volume, all islets were hand picked, followed by a measurement of the diameters of islets in a 4% aliquot of the islet suspension. For diameter measurements, we applied a dissection microscope with a fluorescent illuminator (Bausch and Lomb BVB-125, and 31-33-66) equipped with an ocular micrometer with an accuracy of 25 μm. The total endocrine volume was calculated by measuring the islet diameters, defined as the mean of 2 axes, and by subsequent calculation of the total volume while assuming the islets to be perfect spheres as described in detail elsewhere.[14]

All surgical procedures were performed under halothane anesthesia.

TABLE 1. Enzymatic Activity of Commercial Collagenases Used for Rat Islet Isolation

Enzyme	Activity
Collagenase (FALGPA hydrolysis)	>2.5
Collagenase (collagen digestion)	>1250
Neutral protease (caseinase)	30–100
Trypsin	<0.05
Clostripain	1–2

Activity is provided by the manufacturer. The activity is defined on the basis of more than 2000 islet isolations with more than 50 different lot numbers of collagenases.

Culture of Islets

In the present study, islets were cultured in nontreated Petri dishes (Greiner, Alphen a/d Rijn, The Netherlands) in portions of 4 islets/cm^2 (or as otherwise specified) in glucose-free RPMI1640 (containing 10% fetal calf serum (FCS) (Gibco, Breda, The Netherlands) or CMRL1066 (containing 10% FCS (Gibco) at 37°C in humidified air containing 5% CO_2. Both media were supplemented with appropriate amounts of glucose. Before testing, islets were collected by

hand picking under a dissection microscope with a fluorescent illuminator.

Insulin Secretion During Glucose Challenge

In static incubation experiments, islets were tested in 4 separate samples of 10 islets each. To minimize the variability of the mean insulin responses, we meticulously selected islets with diameters between 150 and 200 μm. The islets were preincubated for 45 minutes in 2 mL Krebs-Ringer Bicarbonate (KRB), gassed with 95% O_2 and 5% CO_2, containing 0.25% BSA and 2.75 mmol/L glucose. The quantitative insulin secretion was then assessed by 3 consecutive incubations of (1) 45 minutes in 2.75 mmol/L glucose in KRB, (2) 45 minutes in 16.5 mmol/L glucose in KRB, and (3) 45 minutes in 2.75 mmol/L glucose in KRB. At the end of each incubation, the incubation media were removed and frozen for insulin determination. The insulin secretory responses were expressed as nanograms of insulin $\cdot$ mL^{-1} $\cdot$ 10 $islets^{-1}$ $\cdot$ 45 $minutes^{-1}$.

Statistical Analysis

Results are expressed as mean ± SEM. Statistical comparisons were made with the Mann-Whitney *U* test. A *P* value <0.05 was considered statistically significant.

RESULTS

Effect of Islet Donor Characteristics

To test the effect of the rat strain on the yield of functional islets, we applied AO/G, Wistar/G, and Lewis rats since these strains have been successfully used in our previous studies on islet isolation[14–17] and transplantation. Figure 1 shows a typical batch freshly isolated from the rat. In batches of fresh islets, we compared the number, volume, and function of islets isolated from the 3 rat strains in 1 islet isolation procedure using the same islet isolation chemicals. This experiment was repeated 6 times. This comparative study of the islet number from the 3 rat strains showed that the AO/G rat was associated with an almost twofold higher yield ($P < 0.02$) in the number of islets than the Wistar/G and Lewis rats. The concomitant calculated endocrine volume of the isolated islets showed a less dramatic difference in yield since the AO/G rat showed an endocrine volume that was not twofold but only 20% higher than the yield from the Wistar/G and Lewis rats (Table 2).

The more pronounced difference in number than in volume of the islets is the consequence of the difference in size of the islets after isolation since Wistar/G and Lewis rats contained statistically significantly more islets with a diameter of ≥180 μm (*P* always <0.05) and significantly fewer islets with a diameter of ≤120 μm (*P* always <0.05) than AO/G rats (Fig. 2).

Not only the yield but also the function of the islets was dependent on the rat strain used. Basal insulin secretion (during challenge with 2.75 mmol/L glucose) was lower from Wistar/G islets than from AO/G ($P < 0.02$) and Lewis islets ($P < 0.02$). Stimulated insulin secretion (during challenge with 16.7 mmol/L glucose) was higher from Lewis islets than from Wistar ($P < 0.05$) and AO/G ($P < 0.018$) islets (Fig. 3). The observation that AO/G rats gave the highest yield but not the highest glucose-induced insulin response illustrates that a high yield is not necessarily associated with a higher glucose-induced insulin response.

To investigate whether the weight of the animals (ie, age) influences the isolation of functional islets, we repeated the above experiments with animals weighing 240–260, 300–320, and 350–370 g. We have restricted these experiments to AO/G rats since these animals showed the highest yields.

As shown in Table 3, the number of islets obtained from the rats weighing 300–320 g ($P < 0.015$) and 350–370 g ($P < 0.01$) was higher than that isolated from rats weighing 240–260 g. Donation of islets was similar and not statistically different from the rats weighing 300–320 and 350–370 g. When comparing the calculated endocrine volume of the isolated islets, we found a weight-dependent and statistically significant increase in yield (*P* always <0.05). This was caused by both an increase in size of islets and an increase in the number of islets yielded when AO/G rats become larger in weight (Fig. 4).

The glucose-induced insulin response was similar in islets obtained from animals weighing 240–260 and 300–320 g,

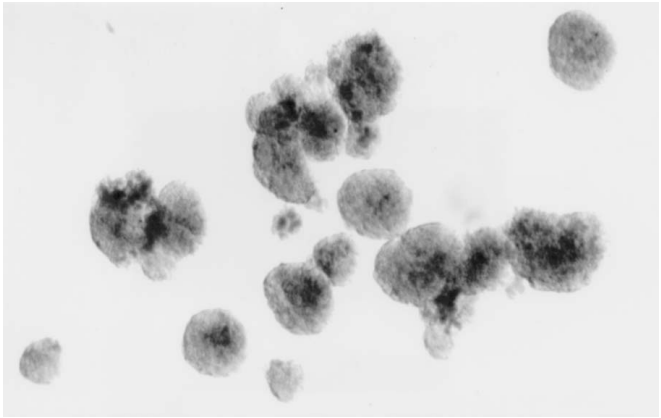

FIGURE 1. Batch of freshly isolated AO/G rats islets (original magnification ×50).

TABLE 2. Number and Endocrine Volume of Islets Isolated From AO/G, Wistar/G, and Lewis Rats

Rat Strain	No. of Rats	No. of Islets	Endocrine Volume (μL)
AO/G	6	1117 ± 109	9.8 ± 1.2
Wistar/G	6	612 ± 74	7.7 ± 0.8
Lewis	6	573 ± 102	8.2 ± 0.6

Values represent mean ± SEM.

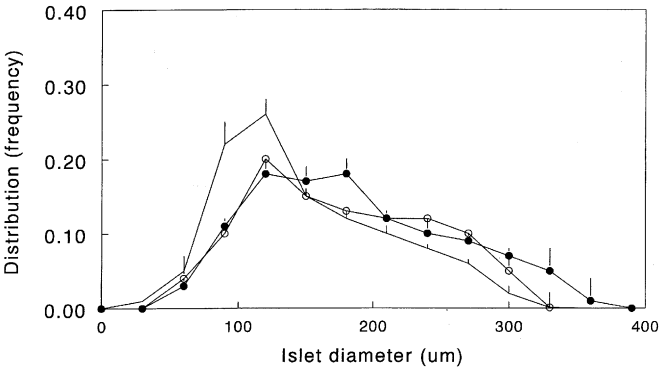

FIGURE 2. Distribution of the islet diameters after isolation of islets from AO/G (no symbol), Wistar/G (○), and Lewis rats (●). Values represent mean ± SEM.

while islets obtained from rats weighing 350–370 g showed an elevated basal insulin secretion and a decreased stimulated insulin secretion (P always <0.05) (Fig. 5).

Effect of BSA Source

As shown in a previous study,[3,11] not only the activity of exogenous collagenase but also the deleterious effects of endogenous proteolytic enzymes released during the digestion of the exocrine pancreas can influence the yield of islets during the isolation process. This endogenous proteolytic enzyme activity is suppressed by the addition of BSA.[11,18] Since we repeatedly found with identical collagenases and similar animals sources variations in yield, we questioned whether differences in BSA preparations influenced the islet isolation process.

When comparing the efficacy of 3 different types of BSA in isolation of AO/G rat islets, we found a clear-cut difference in both the yield and the function of the islets (Table 4, Fig. 6). Sigma lot number 26H1012 showed the highest yield and adequate function. The yield and function were somewhat reduced but not statistically significant when the BSA lot number 51K1339 was used. Serva lot number 14177 was associated with a twofold lower yield and inadequate islet function when compared with Sigma lot number 51K1339 and Sigma lot number 26H1012 (P always <0.05).

TABLE 3. Number and Endocrine Volume of Islets Isolated From AO/G Rats Weighing 240–260 g, 300–320 g, and 350–370 g

Weight (g)	No. of Rats	No. of Islets	Endocrine Volume (µL)
240–260	6	792 ± 67	6.9 ± 0.9
300–320	6	1080 ± 127	10.4 ± 0.8
350–370	6	1102 ± 98	13.2 ± 1.2

Values represent mean ± SEM.

Effect of Culture Conditions

We determined the optimal culture conditions for isolated rat islets since this is still matter of some dispute.[5,19–22] Therefore, we compared the function of the islets in 2 commonly used culture media (ie, CMRL 1066 and RMPI 1640) with varying glucose concentrations and we determined the optimal density of islets per square centimeter of the culture flask.

As shown in Figure 7, the glucose-induced insulin response of cultured rat islets was always higher in CMRL1066 than in RPMI1640. Culture had no effect on the function of the islets until day 5 of culture since we observed no statistical significant reduction in glucose-induced insulin response of the islets. However, at day 7, we found a reduction of 40% in glucose-induced insulin response of the islets. This reduction was similar in CMRL1066 and RPMI1640.

The glucose concentration in the culture medium did not have a more than a mild effect on the glucose-induced insulin response. In CMRL1066, we only observed a statistical sig-

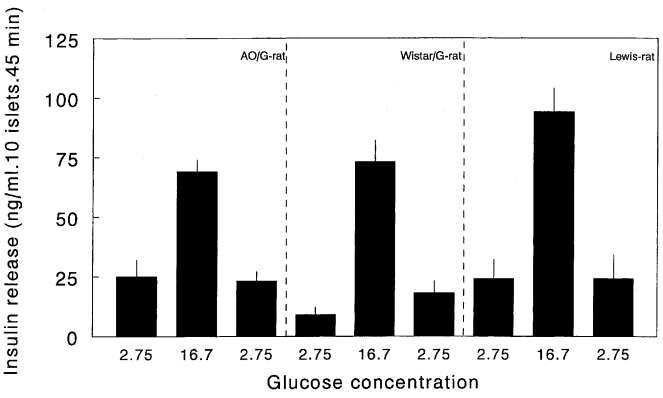

FIGURE 3. Glucose-induced insulin response of isolated AO/G, Wistar/G, and Lewis rat islets. Values represent mean ± SEM.

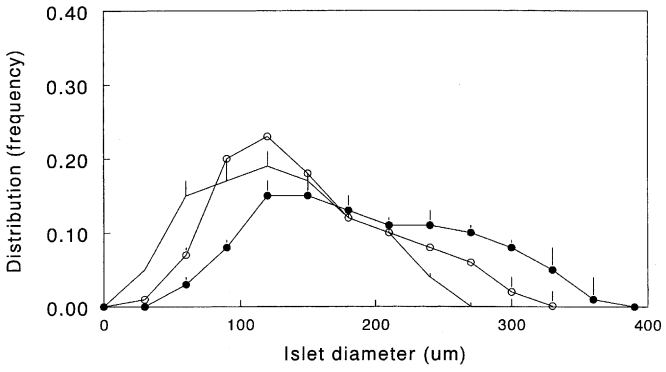

FIGURE 4. Distribution of the islet diameters after isolation of islets from AO/G rats weighing 240–260 g (no symbol), 300–320 g (○), and 350–370 g (●). Values represent mean ± SEM.

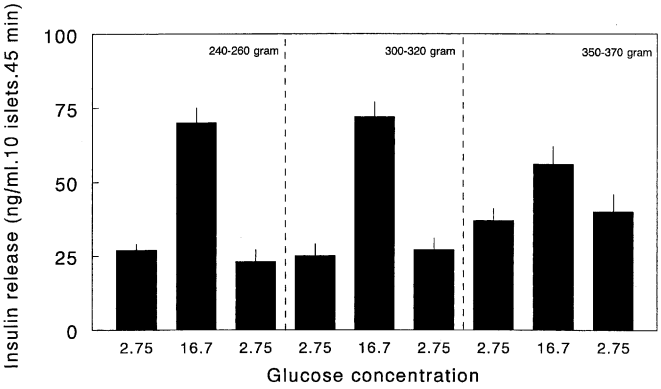

FIGURE 5. Effect of rat donor weight on the glucose-induced insulin response of isolated AO/G rat islets. The rats weighed 240–260, 300–320, or 350–370 g. Values represent mean ± SEM.

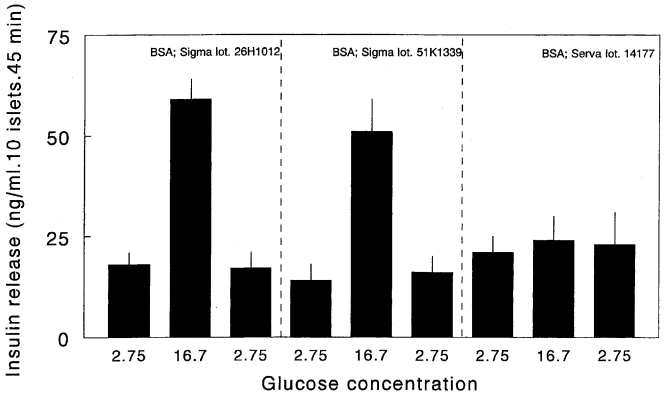

FIGURE 6. Effect of types of BSA on the glucose-induced insulin response of isolated AO/G rat islets. The types of BSA used were Sigma lot numbers 26H1012 and 51K1339 and Serva lot number 14177. Values represent mean ± SEM.

nificant difference at day 2, where islets in CMRL1066 with 8.3 mmol/L glucose showed a higher glucose-induced insulin response than islets in CMRL1066 with 11.1 mmol/L glucose. With RMPI 1640, we found a statistically significant difference at day 5, where islets in RPMI1640 with 11.1 mmol/L glucose (ie, the conventional glucose concentration) showed a much lower glucose-induced insulin response than islets in RPMI1640 with 5.5 and 8.3 mmol/L glucose. In view of the results obtained above, we used CMRL1066 with 8.3 mmol/L glucose as the standard culture medium in the subsequent experiments.

Conventionally, we culture islets at a density of 4 islets per square centimeter of culture flask. Since increasing the density of islets can elevate the capacity of culture, we compared the number and function of islets at several time points after culture at densities of 4, 12, and 24 islets per square centimeter.

As shown in Figure 8, culture was always associated with a reduction in the number of islets. This reduction was much greater with the higher densities of 12 and 24 islets per square centimeter than with the conventional 4 islets per square centimeter. The effect of islet density on islet yield was most pronounced after 7 days of culture where 58 ± 2% survived the culture procedure at an islet density of 4 islets per square centimeter and only 23 ± 3% and 21 ± 3%, respectively, were found in cultures of 12 and 24 islets per square centimeter. The applied islets in the above-described experiments were free of any contamination by exocrine tissue. In some instances, we applied islets with remnants of exocrine tissue in the cultures and found an even more dramatic decrease in number of islets during culture.

This loss of islets when cultured at higher densities was mainly caused by fusion of islets rather than by disintegration since we observed more large islets and islets in the process of fusion when cultured at densities of 12 and 24 islets per square centimeter. This fusion of islets was not without consequence for the function of the islet tissue since, after 7 days of culture, islets from 12 and 24 islets per square centimeter showed a statistically significantly lower glucose-induced insulin response than islets from cultures of 4 islets per square centimeter (Fig. 9).

TABLE 4. Number and Endocrine Volume of Islets Isolated From AO/G With Application of 3 Different BSA Sources*

BSA Lot Number	No. of Rats	No. of Islets	Endocrine Volume (μL)
Sigma 26H1012	4	1089 ± 102	9.6 ± 1.6
Sigma 51K1339	4	898 ± 192	8.9 ± 1.7
Serva 14177	4	467 ± 206	5.1 ± 2.1

Values represent mean ± SEM.

DISCUSSION

Isolation of functional islets is essential for adequate performance of studies on islets both in vitro and in vivo.[13,20,27,34,39] Islet isolation yield and islet function depend on many factors,[3–5,23] of which only a few have been identified. In the present study, we show that the strain and weight of the rat donor, the variations in BSA source, and the culture conditions of islets are pertinent factors in efficacious collection of islets for transplantation studies.

The rationale for the present study was based on previous studies in which we found large variations in function and yield when we used different rat strains or when new batch numbers of chemicals for isolation and culture were applied. These variations were as such that we were reluctant to com-

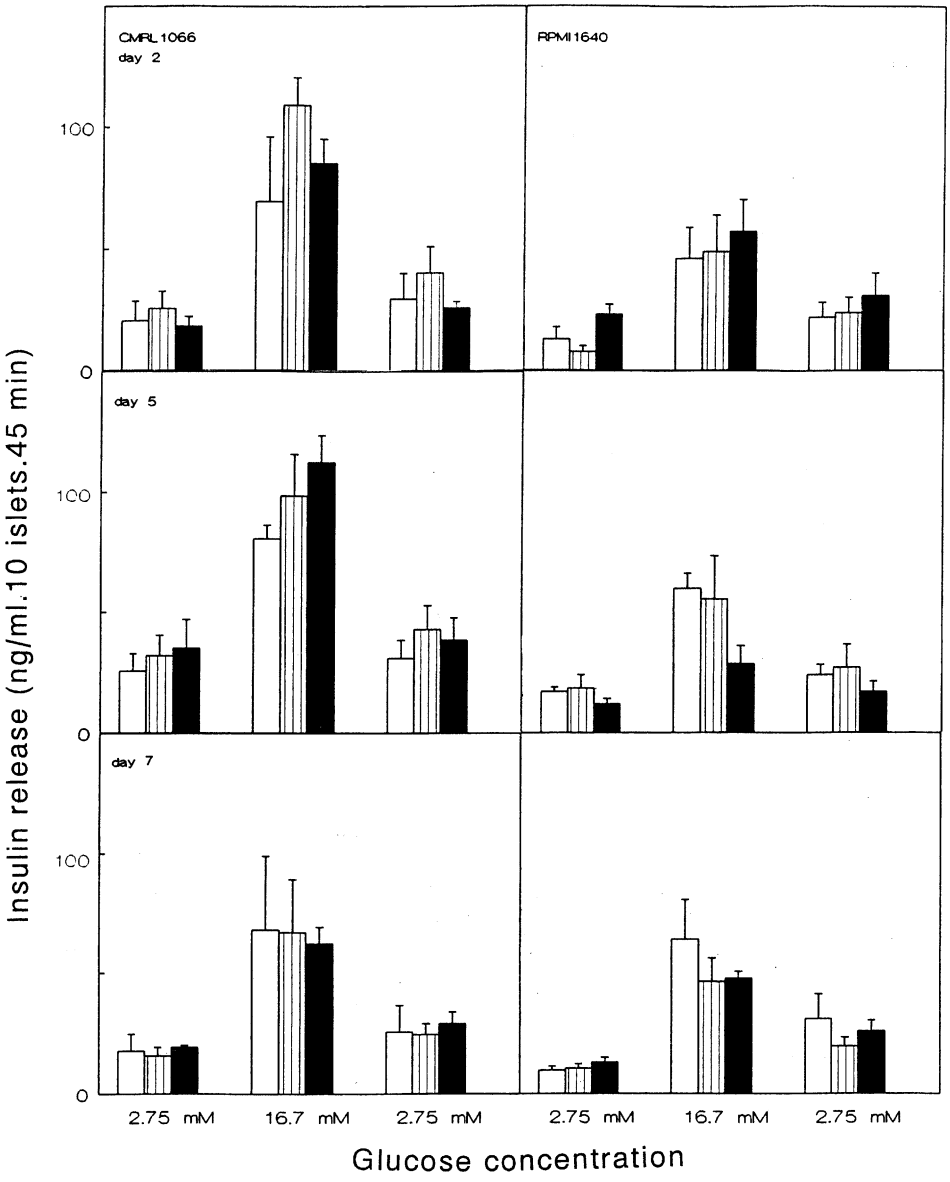

FIGURE 7. Effect of medium type and glucose concentration on the glucose-induced insulin response of isolated AO/G rat islets at days 2, 5, and 7 of culture. The open columns represent the responses of islets cultured in medium containing 5.5 mmol/L glucose, the striped columns represent the responses of islets cultured in medium containing 8.3 mmol/L glucose, and the solid columns represent the responses of islets cultured in medium containing 11.1 mmol/L glucose. Values represent mean ± SEM.

pare studies when one of these factors varied. The present study shows that AO rats yield almost twice the number of islets as compared with Wistar and Lewis rats. However, when the endocrine volume was compared, we found a less pronounced effect of animal strain, which can be attributed to the relatively large numbers of islets with a small volume of the yielded AO islets. This latter observation is pertinent since it implies that there is no reason to reject Wistar and Lewis rats as islet donors in transplantation studies since the islet endocrine volume and not the islet number determines the success of islet grafts.[24] However, for in vitro studies in which numbers rather than endocrine volume is of importance, it is advisable to use AO/G rats since they are associated with a twofold reduction in the number of experimental animals required.

Our observation that heavier rats yield more islets, and a higher endocrine volume should not be interpreted as an indication to use animals with higher weights for islet studies since islets from larger animals show a decreased and inadequate glucose-induced insulin response. This decrease in insulin response of islets from heavier animals may well be the consequence of increased insulin resistance in rats with a higher weight.[25–27] Insulin resistance is known to induce a larger metabolic load on the islets with growth of the tissue[25,28] and, in severe cases, dysfunction of the tissue as a consequence.[25,27,29]

In previous studies,[3,18] it has been shown that endogenous proteolytic activity released during the dissociation process exerts a deleterious effect on the isolation procedure. The

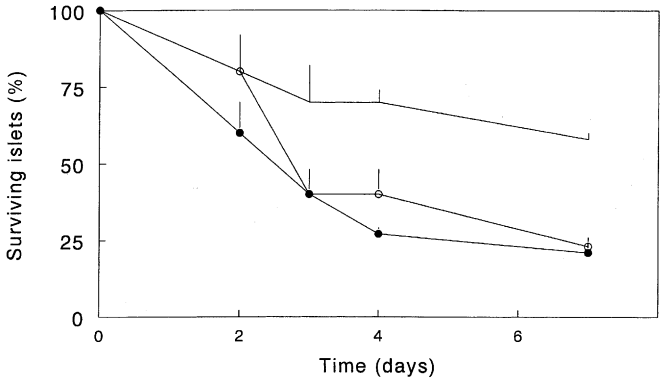

FIGURE 8. Effect of the number of islets per square centimeter of culture flask on the percentage of harvested islets at days 2, 3, 4, and 7 of culture. Surviving islets were determined by counting the islets on the indicated days. Cultures were started with 4 islets per square centimeter (no symbol), 12 islets per square centimeter (○), or 24 islets per square centimeter (●). Cultures were always performed with AO/G islets in CMRL1066 with 8.3 mmol/L glucose. Values represent mean ± SEM.

proteolytic activity in the dissociation medium can cause rupture of the cell, release of DNA, and can consequently interfere with the isolation process. Adding protease inhibitors to the isolation media such as BSA can prevent cell lysis and can therefore prevent release of proteolytic enzymes.[3] Surprisingly, we found that this beneficial effect of BSA on islet isolation is dependent on the type and batch of BSA used. For unknown reasons, we found an almost twofold decrease in islet yield when Serva BSA was used instead of Sigma BSA. These results suggest that it is advisable to test the efficacy of BSA preparations in suppressing proteolytic activity before use in islet isolation.

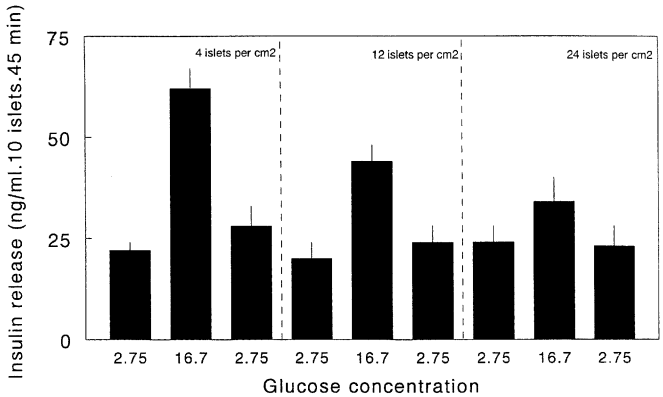

FIGURE 9. Effect of the number of islets per square centimeter of culture flask on the glucose-induced insulin response of isolated AO/G rat islets at day 7 of culture. Cultures were always performed with AO/G islets in CMRL1066 with 8.3 mmol/L glucose. Values represent mean ± SEM.

A pertinent procedure after enzymatic isolation of islets is culture of the tissue. This culture step is performed for various reasons: to create the option for safety screening, to collect sufficient tissue for transplantation, to allow disintegration of contaminating exocrine tissue, or to allow the islets to recover from damage of the enzymatic isolation.[4,5,21] Selection of appropriate media has been shown to depend on the animal source of islet isolation.[19] For culture of rat islets, 2 media are commonly used, CMRL1066 and RPMI1640.[1–3,12,16] In the present study, we show that CMRL1066 is preferred for culture since it is associated with higher insulin secretory capacity of the islets. Another reason to prefer CMRL1066 over RPMI1640 for islet culture before transplantation has been the observation that CMRL1066 is associated with a decrease in alloreactivity since immune cells such as dendritic cells and endothelial cells do survive for only a short period of time in CMRL1066.[30–32]

To our knowledge, we are the first to report on the effect of numbers of islets per square centimeter on islet function and survival in culture. We show that higher numbers of islets per square centimeter of culture flask are associated with a deleterious effect on number and function of islets. During our studies, we found that this effect was even more pronounced when islets contained remnants of exocrine tissue. This has serious consequences for transplantation studies since it implies that, depending on the quality of the yielded islets, a subjective, adapted judgment must be made for the culture conditions. With completely clean islets (ie, the absence of remnants of exocrine tissue on the islets), it is advisable to culture not more than 4 islets per square centimeter. This implies that for 1 transplantation experiment for which 4000 islets are required,[3,24] a total of 1000 cm^2 is required. This equals 7 large culture flasks of 162 cm^2. Obviously, this is laborious and impractical, and thus not many laboratories use these low numbers of islets per square centimeter.

Again, our findings emphasize that selection of optimal circumstances of the islet isolation process and identification of pertinent factors determining success and failure of the procedure are required for efficacious isolation of pancreatic islets and, consequently, the maximum chance for success of the islet grafts.[3,7,16,33–35]

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