Improved Human Islet Isolation Using a New Enzyme Blend, Liberase

Elina Linetsky, Rita Bottino, Roger Lehmann, Rodolfo Alejandro, Luca Inverardi, and Camillo Ricordi

Enzymatic digestion of donor pancreases is a vital step in human and large mammalian islet isolation. The variable enzymatic activities of different batches of commercially available collagenase is a major obstacle in achieving reproducibility in islet isolation procedures. In the present work, the effectiveness of Liberase, a standardized mixture of highly purified enzymes recently developed for the separation of human islets, was compared with that of a traditional collagenase preparation (type P). The results of 50 islet isolations using Liberase enzyme were compared with those of 36 isolations with collagenase, type P. No significant differences in donor age, cold ischemia time, digestion time, or weight of the pancreases were observed between the two groups. Islet yield was significantly higher in the group where the Liberase enzyme was used. All parameters examined (islet number, islet number per gram of tissue, islet equivalent number, and islet equivalent number per gram of tissue) were significantly improved when Liberase enzyme was used. Different lots of Liberase enzyme were tested, and no difference was observed. Islets isolated with Liberase enzyme were also of larger size and were much less fragmented, suggesting a gentler enzymatic action and better preservation of anatomical integrity. Islets isolated with Liberase enzyme, assessed both in vitro and in vivo, revealed a functional profile similar to that of islets separated with collagenase. Liberase enzyme appears, therefore, to represent a new powerful tool for improving the quality of human islet isolation. *Diabetes* **46:1120–1123, 1997**

The implementation of clinical trials of islet transplantation requires improved procedures for isolation of large numbers of islets (1–3) and their separation from the exocrine tissue with the highest degree of purity (4). Improvements in the islet isolation techniques and the availability of standardized reagents, especially the ones involved in the enzymatic digestion of the gland, would represent significant advances toward this goal (5,6). Collagenase has been the preparation of choice in the digestion of the pancreas, since it aids in rapid dissociation of the stromal component of the gland, while preserving the anatomical integrity of the endocrine tissue. Several problems, however, have characterized the use of conventional collagenase preparations in the past years, including the irreproducibility of enzymatic action among different commercially available sources and even different lots. Recently, Liberase, a standardized mixture of highly purified enzymes, was made available, which offered a seeming advantage of improving the reproducibility of islet isolations from lot to lot (7). Liberase purified enzyme blend is comprised of highly purified collagenase isoforms I and II from *Clostridium histoliticum*, and Thermolysin from *Bacillus thermoproteolyticus*. The formulation of these components is specifically optimized for human islet isolation and is the subject of a patent application filed by Boehringer Mannheim (8).

The purpose of the present study was to evaluate the efficacy by assessing parameters, such as islet number (IN), islet equivalent number (IEQ), IN per gram of tissue, and IEQ per gram of tissue, of the new formulation in human islet isolation and to compare it with that of an available conventional collagenase preparation (type P). We have, therefore, compared the results of 50 islet isolations using Liberase enzyme with 36 isolations performed with collagenase. While type P collagenase was always obtained from a selected single lot (lot 64) that had proven, in our experience, to give the most successful and reproducible results, five different commercially available lots of the Liberase enzyme were studied and compared both with one another and with collagenase, type P.

In summary, our results suggest that Liberase enzyme represents an advantageous alternative to collagenase. The high reproducibility of the results, compared with the frequent lack of reproducibility recorded with various lots of collagenase, represents a further advantage characterizing the new enzyme formulation, in terms of eliminating the need of prescreening of collagenase lots.

RESEARCH DESIGN AND METHODS

Donors. Human pancreases were procured by the United Network for Organ Sharing and obtained from multi-organ heart-beating donors between the ages of 18 and 55 years. The organs were obtained after in situ perfusion of the abdominal aorta with cold University of Wisconsin solution and kept on ice before processing. Pancreases with cold ischemia time (CIT) longer than 24 h were excluded from this study.

Islet isolation. To separate pancreatic islets from human pancreases, we used a modification of the automated method (5,6) for human islet isolation. Digestion was performed with either Liberase enzyme (lots 14365720, 1427420, GLA 102, GLA 103, GLA 104) or collagenase, type P (lot 64, both from Boehringer Mannheim, Indianapolis, IN), at concentrations of 1.4 and 2.8 mg/ml, respectively. The digestion process was allowed to proceed for ~23 min. The tissue was then collected, pelleted by centrifugation, and purified using discontinuous EuroFicoll (Fresenius, Walnut Creek, CA, and Sigma, St. Louis, MO) gradients (9) and a COBE rotor (10). Islets were counted manually and scored for size. An algorithm was used for the calculation of IEQ (11).

From the Diabetes Research Institute (E.L., R.B., R.L., L.I.), University of Miami School of Medicine; and the Miami Veterans Administration Medical Center (R.A., C.R.), Miami, Florida.

Address correspondence and reprint requests to Dr. Camillo Ricordi, Diabetes Research Institute, 1450 NW, 10th Ave. (R-134), Miami, FL 33136. E-mail: cricordi@mednet.med.miami.edu.

Received for publication 10 January 1997 and accepted in revised form 12 March 1997.

CIT, cold ischemia time; IEQ, islet equivalent number; IN, islet number; STZ, streptozotocin.

Functional study. Static incubation of hand-picked islets at different glucose concentrations was performed to evaluate islet response to glucose challenge. Briefly, triplicates of 50 hand-picked islets were incubated at 37°C in Krebs-Ringer bicarbonate buffer containing 1.67 mmol/l glucose for 1 h, followed by a 2nd hour of incubation with 16.7 mmol/l glucose (1,12). Release of insulin in the supernatants was assayed using a radioimmunoassay.

Transplantation. In vivo evaluation of the islets isolated with Liberase enzyme was performed by transplantation of 800 IEQ under the kidney capsule of 8-week-old Balb/c$^{nu/nu}$ mice (Charles River, Wilmington, MA) (13–16). Mice were rendered diabetic by a single intravenous injection of streptozotocin (STZ), 220 mg/kg (Sigma, St. Louis, MO) via the tail vein. Only mice with nonfasting blood glucose values >400 mg/dl were used as transplant recipients. After islet transplantation, blood glucose levels were monitored every 3 days using an Accu-Chek Easy blood glucose meter (Boehringer Mannheim). Thirty days after transplant, the islet-bearing kidney was removed, and mice were monitored to confirm a prompt return to a hyperglycemic state.

Statistical analysis. Analysis of data distribution and comparison among groups was performed with Statistica for Windows statistical software (StatSoft, 1993; Tulsa, OK). Available data from both groups were analyzed by Student's *t* test; $P < 0.05$ was considered the cut-off for statistical significance. Correlation among the variables was estimated with Pearson's correlation coefficients. Multiple step-wise regression analysis was performed to assess the contribution of different variables to the islet yield.

RESULTS

Analysis of islet yield. First, we compared the absolute islet yield obtained in the two groups (Liberase enzyme and collagenase, type P). IN was significantly different, with 398,840 ± 217,405 islets isolated per pancreas in the Liberase enzyme group versus 260,605 ± 132,283 in the collagenase group ($P < 0.001$) (data not shown). Although no significant difference was found when weight of pancreases was analyzed in the two groups, a more precise evaluation of the islet yield was undertaken by calculating the IN per gram of processed tissue. This comparison also clearly indicated (Fig. 1) that the islet yield was significantly improved in the Liberase enzyme group (5,509 ± 3,171) as compared with that of the collagenase group (3,327 ± 2,022, $P < 0.001$). Next, to assess islet size, islets were counted and scored according to diameter. Islet number in different diameter classes was expressed as IEQ by means of relative conversion into islets with a diameter of 150 μm. By calculating IEQ, therefore, information was obtained on both number and size of the isolated islets. Islets purified with Liberase enzyme averaged 487,438 ± 343,922 IEQ per pancreas isolation, while islets purified with collagenase averaged 259,167 ± 150,152 IEQ (data not shown, $P < 0.001$). As expected (Fig. 1), IEQ per gram of tissue also proved to be significantly different in the Liberase enzyme (6,698 ± 4,920) compared with the collagenase group (3,245 ± 2,041, $P < 0.001$). While IN and IEQ in the collagenase group are very similar, a sharp difference exists between IN and IEQ in the Liberase enzyme group, where IEQ is much higher than IN, suggesting that islet size in this group is, on average, larger (Fig. 2). This is confirmed by analyzing the percentage of islets belonging to different size categories in the two groups (Fig. 2). Lastly, when the parameters described above were analyzed in islet isolations performed with five different lots of Liberase enzyme, no significant variations were observed, unlike what is reported when different lots of collagenase are compared (data not shown).

Analysis of variables. When variables such as CIT, digestion time, weight of the pancreases, and age of the donor, known to affect islet yield, were compared, the only variable significantly different between the two groups was CIT. Average CIT was, in fact, longer in the collagenase group (Table 1). To exclude that the observed prolonged CIT of the pancreases randomly assigned to the collagenase group might account for the lower cell yield, we performed multiple step-wise regression analysis of the indicated variable. Such analysis showed that no significant correlation existed between CIT and islet yield, seemingly ruling out the influence of this variable on the isolation outcome. All other variables analyzed also indicated that no correlation existed, with the obvious exception of the type of enzyme (data not shown).

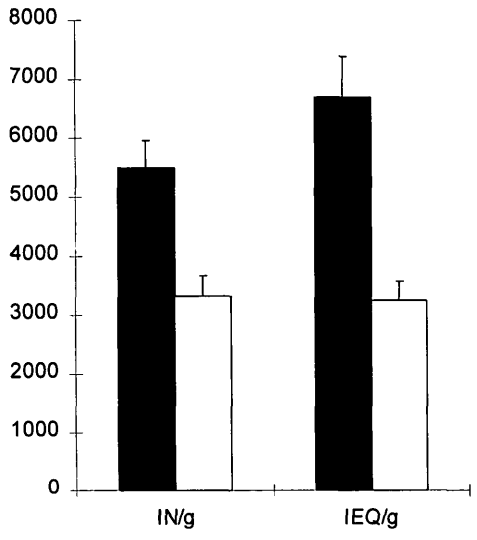

FIG. 1. Islet yield in the pancreases processed with Liberase (■) is expressed in IN per gram of tissue and IEQ per gram of tissue and compared with the average yield of glands processed with collagenase (□). Data are expressed as means ± SD.

In vitro and in vivo studies of islet function. To ascertain that the islets isolated with Liberase enzyme were viable and functionally competent, we studied insulin release in a static incubation assay. The results (Table 2) demonstrate a functional profile of glucose sensitivity, which is comparable with that reported when more conventional enzymes were used during the digestion phase (17). Transplantation of 800 IEQ under the kidney capsule of STZ-induced diabetic nude mice

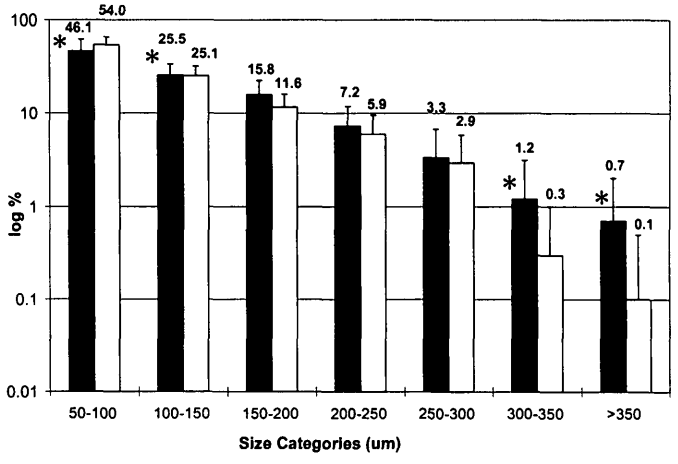

FIG. 2. Islets obtained with Liberase (■) or collagenase, type P (□), were counted and assigned to the indicated size categories. The figure shows the percentage of the total islet count in various categories. *$P < 0.005$.

TABLE 1
Analysis of variables capable of affecting islet cell yield in the two indicated groups

	Type P, lot 64	Liberase	*P*
Donor age (years)	30.3 + 12.6	36.0 + 13.6	NS
CIT (h)	11.2 + 5.1	8.8 + 3.9	0.03
Digestion time (min)	24.4 + 10.0	23.6 + 5.6	NS
Weight (g)	86.9 + 29.0	78.3 + 25.0	NS

Data are means ± SD.

TABLE 2
Static glucose challenge of freshly isolated islets obtained after digestion with Liberase

	1st hour (1.67 mmol/l glucose)	2nd hour (16.7 mmol/l glucose)	Increase ratio (16.7/1.67 mmol/l)
Insulin release ($\mu U \cdot ml^{-1} \cdot islet^{-1} \cdot h^{-1}$)	11.15 ± 1.8	70.9 ± 11.3	6.09 ± 1.2

Data are means ± SD. n = 8.

reversed hyperglycemia and stably maintained normal glucose levels. Thirty days after transplantation, nephrectomy performed on the transplanted animals promptly reestablished a hyperglycemic state (Fig. 3).

DISCUSSION

Variation in islet yield has been traditionally attributed to the variability that exists among different commercially available lots of collagenase. Recently, a new formulation has been introduced—the Liberase enzyme, which largely obviates to the lack of reproducibility. The purpose of this study was to evaluate the use of Liberase enzyme as a potential alternative to type P collagenase in human pancreatic islet isolation. The results obtained from 50 human pancreases in the case of Liberase enzyme and 36 in the case of collagenase, type P, were analyzed. Only one single lot (lot 64) of collagenase, type P, was compared with several different lots of Liberase enzyme. Our results demonstrate that the use of Liberase enzyme not only yields significantly higher absolute IN and IN per gram of tissue, but also allows to recover islets of larger size then those obtained with collagenase, type P. This might be explained by postulating an improved preservation of the anatomical integrity of the endocrine component of the pancreas as a consequence of a less disruptive enzymatic action of Liberase enzyme in comparison with collagenase, type P. Preservation of the structure of the larger islets without their partial digestion and/or fragmentation is a likely explanation for the observed improvements in islet size and, therefore, IEQ and IEQ per gram of tissue. When five different commercially available lots of Liberase enzyme used in this study were compared with one another, no significant variability was observed in the final islet yield. Multiple step-wise regression analysis of variables known to be capable of affecting islet yield, such as CIT, digestion time, donor age, and weight of the pancreases, was performed, in which the influence of these variables along with the type of enzyme used were investigated. All the variables showed normal distribution, and the only two that influenced the final islet yield were the weight of the pancreases and the type of enzyme used. Because the weight is not different between the two groups, the only variable able to contribute to the differences in cell yield is the type of enzyme. CIT, on the other hand, was the only variable analyzed that was different in the two groups. However, multiple regression analysis showed that no significant correlation could be found between CIT and cell yield. Thus, when all the other variables, which do not explain a significant portion of the variance, are controlled for and the isolation technique is standardized, the only variable that provides a significant contribution to the differences in islet yield observed is the type of enzyme used.

Assessment of the islets recovered with Liberase enzyme indicated that their in vitro glucose sensitivity was preserved. Transplantation of these islets into STZ-induced diabetic nude mice was able to reverse and normalize the blood glucose levels providing evidence for in vivo function.

Our results suggest that Liberase enzyme represents an attractive alternative to collagenase, type P, and likely an advantageous one in terms of islet yield. Furthermore, when the minimum degree of variability among Liberase enzyme lots is taken into consideration and compared with the frequent lack of reproducibility recorded with different lots of type P collagenase, an additional important advantage provided by the new enzyme formulation is underlined. This advantage becomes even more prominent when considering costs and time involved in the usual prescreening process of different batches of collagenase.

ACKNOWLEDGMENTS

This work was supported in part by grant DK25802 from the National Institutes of Health, by grant 196102 from the Juvenile Diabetes Foundation, and by a grant entitled "Pre-

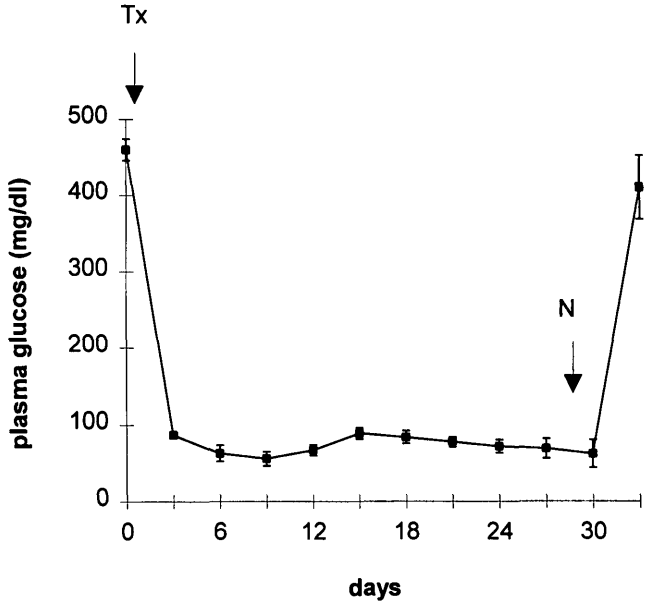

FIG. 3. Glycemic profile of nude mice (n = 4) that received human islets of Langerhans under the kidney capsule. Islet-bearing kidneys were removed 30 days after transplantation to ascertain whether the observed normalization of the glycemic profiles could be causally linked to the implanted islets. Data are expressed as means ± SD. Tx, transplantation; N, nephrectomy.

venting early loss of transplanted islets" from the American Diabetes Association.

REFERENCES

1. Gray DWR, McShane P, Grant PJ: A method for isolation of islets of Langerhans from the human pancreas. *Diabetes* 33:1055–1061, 1984
2. Hering BJ, Bretzel RG, Federlin K: Current status of clinical islet transplantation. *Horm Metab Res* 20:537–545, 1988
3. Gray DWR, Morris PJ: Developments in isolated pancreatic islet transplantation. *Transplantation* 43:321–323, 1987
4. Lacy PE, Kstianovsky M: Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes* 16:35–39, 1967
5. Ricordi C: The automated method for islet isolation. In *Pancreatic Islet Cell Transplantation.* Ricordi C Ed. Austin, Texas, RG Landes, 1992, p. 99–112
6. Scharp DW, Lacy PE, Finke E, Olack BJ: Low-temperature culture of human islets isolated by the distension method and purified with Ficoll or Percoll gradients. *Surgery* 102:869–879, 1987
7. Linetsky E, Selvaggi G, Bottino R, Kong SS, Qian T, Alejandro R, Ricordi C: Comparison of collagenase, type P and Liberase during human islet isolation using the automated method. *Transplant Proc* 27:3264, 1995
8. Boehringer Mannheim Corporation: A purified mixture of collagenase and two other proteases obtained from *Clostridium histolyticum.* Patent Application WO 96/00283
9. Ricordi C, Tzakis AG, Carrol PB, Zeng Y, Rilo HLR, Alejandro R, Shapiro R, Fung JJ, Demetris AJ, Mintz DH, Starzl TE: Human islet isolation and allotransplantation in 22 consecutive cases. *Transplantation* 53:407–414, 1992
10. Alejandro RA, Strasser S, Zucker PF, Mintz DH: Isolation of pancreatic islets from dogs: semiautomated purification on albumin gradients. *Transplantation* 50:207–210, 1990
11. Ricordi C, Gray DWR, Hering BJ, Kaufman D, Warnock G, Kneteman N, Lake S, London N, Socci C, Alejandro R, Zeng Y, Scharp D, Viviani G, Tzakis A, Bretzel R, Federlin K, Pozza G, James R, Rajotte R, Di Carlo V, Morris P, Sutherland D, Starzl T, Mintz D, Lacy P: Islet isolation assessment in man and large animals. *Acta Diabetol Lat* 27:185–195, 1990
12. Andersson A, Borg H, Groth CG, Gunnarsson R, Hellerstroom C, Lundgren G, Westman J, Ostman J: Survival of isolated human islets of Langerhans maintained in tissue culture. *J Clin Invest* 57:1295–1301, 1976
13. Ballinger WF, Lacy PE: Transplantation of intact pancreatic islets in rats. *Surgery* 72:175–186, 1972
14. Bowen K, Andrus L, Lafferty KJ: Successful allotransplantation of mouse pancreatic islets to nonimmunosuppressed recipients. *Diabetes* 29 (Suppl. 1):98–104, 1980
15. Stock PG, Gores P, Sutherland DER: Renal subcapsular versus portal vein site for islet transplantation in mice. *Diabetologia* 30:193, 1987
16. Ricordi C, Flye MW, Lacy PE: Renal subcapsular transplantation of clusters of hepatocytes in conjunction with pancreatic islets. *Transplantation* 45:1148–1151, 1988
17. Lakey JRT, Warnock GL, Rajotte RV: Comparison of methods used for the removal of DMSO following cryopreservation and the development of an automated protocol. *Cell Transplantation* 6:163–172, 1997