A Major Loss in Islet Mass and B-cell Function Precedes Hyperglycemia in Mice Given Multiple Low Doses of Streptozotocin

VAGN BONNEVIE-NIELSEN, MICHAEL W. STEFFES, AND ÅKE LERNMARK

SUMMARY

Streptozotocin (SZ) given in five low doses causes diabetes and an associated lymphocytic infiltration of the pancreatic islets. Using C57BL/KsJ-mice, we demonstrate a reduction in islet number (−38%) and volume (−64%) within 1 day following the last injection of SZ. A substantial fall of insulin secretory capacity (−84%) in the in vitro perfused pancreas matches the reduction in islet cell volume. The parameters of decreased islet function seem to precede the peak of lymphocytic infiltration, occurring 3 days after the last dose of SZ. These functional changes are readily demonstrable before a rise in fasting blood glucose, but they seem to be reflected more readily by a rise in nonfasting blood glucose levels. With development of overt diabetes, as measured by elevated fasting and nonfasting glucose levels, the measures of islet volume and function are reduced to levels only 1–2% of those found in control mice. Taken together, these observations reflect a rapid, islet-toxic effect of SZ that substantially decreases insulin secretory capacity. When islet function falls more than 90%, blood glucose levels begin to reflect the pathophysiologic process. In many aspects, the low-dose SZ model of diabetes parallels the development of diabetes in man. If so, measures other than blood sugar must be developed to identify at an early stage processes reducing islet volume and function. DIABETES *30*:424–429, May 1981.

At the onset of insulin-dependent diabetes mellitus, there appears to be a considerable reduction in insulin secretion and pancreatic B-cells. It has been suggested from several studies using the C-peptide radioimmunoassay[1,2] that only about 45% of patients with juvenile-onset insulin-dependent diabetes have residual B-cell function.[3] The B-cell function seems to cease completely within 10 yr in the majority of patients. Little is known about the pathologic processes and the time involved to induce a diabetic state, as the diabetic patient is not identified until the time of onset. Recent investigations focus on the possible role of infectious agents or environmental factors operating alone or in conjunction with autoimmune mechanisms in susceptible individuals.

Following five subdiabetogenic doses of streptozotocin in mice, hyperglycemia did not develop until a few days following the last injection.[4] The delayed, progressive increase in plasma glucose was associated with lymphocytic infiltrates of the pancreatic islets and a marked B-cell necrosis.[5,6] Similar morphologic changes have been described in the pancreatic islets of insulin-dependent diabetic patients.[7] The degree of B-cell destruction necessary for a diabetic state to develop in man or experimental animals is not known, nor is it clear to what extent the B-cell function is affected by a possible pathologic process induced by a toxic agent or by the presence of inflammatory cells.

In the present work, we documented substantially reduced insulin-secretory capacity and islet volume and number in the early stages of diabetes induced by multiple low doses of streptozotocin. These alterations occurred before significant elevations in fasting or nonfasting blood glucose levels and before marked lymphocytic infiltration.

MATERIALS AND METHODS

Animals and treatment with streptozotocin. Male, 3-mo-old, C57BL/KsJ-mice (Jackson Laboratories, Bar Harbor, Maine) were given five daily i.p. injections of 40 mg/kg body wt streptozotocin (Upjohn Chemical Company, Kalamazoo, Michigan) dissolved immediately before use in 25 mmol/L sodium citrate buffer (pH 4.5).[4] The first day of streptozotocin administration is designated as day 1 of the study. Thus, day 6 is the first day after the last dose. Controls were given sodium citrate buffer alone. The mice were individually housed, and blood samples, nonfasting and fasting (16–

From the Hagedorn Research Laboratory, DK-2820 Gentofte, Denmark (V B.-N., M.W.S., and A.L.), and the Department of Laboratory Medicine and Pathology, University of Minnesota, School of Medicine, Minneapolis, Minnesota (M.W.S.).

Address reprint requests to Åke Lernmark, Hagedorn Research Laboratory, Niels Steensensvej 6, DK, 2820 Gentofte, Denmark.

Received for publication 13 October 1980.

18 h), were obtained from the retro-orbital plexus.[8] Blood glucose was determined by a glucose oxidase method (Glox Novum, Kabi, Sweden).

Pancreas perfusion. Control or streptozotocin-treated mice were fasted for 16 h and anesthetized by a single peritoneal injection of sodium pentobarbital (80 g/kg body wt). The pancreas was perfused according to the method of Grodsky and Fanska[9] with minor modifications. To obtain a whole organ perfusion both the celiac trunk and superior mesenteric artery were perfused through a catheter placed in the aorta. The perfusate (see below) was pumped through the pancreas at 38°C without recycling by a peristaltic pump (Pharmacia P-3, Pharmacia, Fine Chemicals, Sweden), at a flow of 0.80 ± 0.13 ml/min (mean ± SD for N = 28) and a pressure of 46 ± 10 mm Hg (mean ± SD for N = 29). Viability of the organ block was verified by the continued presence of gastric secretion and duodenal peristalsis. The perfusion apparatus was equipped with two parallel chambers allowing a rapid shift to a new medium.[10,11] The perfusate was a Krebs-Ringer bicarbonate (KRB) buffer with the following composition: 115 mmol/L NaCl, 4.7 mmol/L KCl, 2.56 mmol/L $CaCl_2$, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4$, and 20 mmol/L $NaHCO_3$. The KRB buffer was supplemented with 4% bovine serum albumin, fraction V (Miles Laboratories Inc., Elkhart, Indiana). The perfusate was filtered through a Millipore (0.22 μm) filter, and the pH was kept at 7.4 by equilibrating the medium to O_2/CO_2-95%/5% by means of a roller, continuously rotating in each chamber.

In each experiment, the pancreas was first perfused for 20 min in perfusate containing 3 mmol/L D-glucose and then for 47 min with 30 mmol/L D-glucose. Samples, collected in a fraction-collector, were frozen immediately and stored at −20°C. Insulin was determined by radioimmunoassay; bound and free hormones were separated by ethanol precipitation.[12] A 0.04-mol/L phosphate buffer (pH 7.4), containing 0.6 g/L NaCl and 1 g/L human serum albumin, was used to dilute samples and reagents. Insulin was iodinated with chloramine-T as the oxidizing agent, and polyacrylamide gel electrophoresis was used to purify the tracer.[13] Antibodies against porcine insulin were raised in guinea pigs. A guinea pig anti-porcine insulin antiserum (Antiserum GP12) and crystalline mouse insulin as the standard (Novo Research Institute, Copenhagen, Denmark) were used.

Streptozotocin was injected on days 1–5 and perfusion of the isolated mouse pancreas was done on days 6, 8, 12, and 15. Results are expressed as ng insulin release per min and shown as mean values ± SEM.

Determination of islet volume. Estimation of islet volume was performed by staining the mouse pancreas with dithizone and determining the area of the stained islets by quantitative morphometry. Following perfusion of the mouse pancreas with 30 mmol/L D-glucose, KRB buffer supplemented with 3 mmol/L D-glucose, 7.1% ethanol, 500 K I U/ml Aprotinin (Novo A/S, Copenhagen, Denmark), 1 g/L procaine hydrochloride, and 1.24 mmol/L dithizone (Merck, Darmstadt, Germany) was infused via a side-arm syringe for 3 min. Procaine hydrochloride was added to dilate the vessels, since preliminary studies have shown that without procaine hydrochloride, the pressure increased 5–10 mm Hg in response to dithizone-containing KRB. The pancreas, after exposure to the stain, was perfused for 5 min with KRB supplemented with 5 mmol/L D-glucose, resulting in a washout of the stain from the blood vessels, leaving the islets stained red.

The organ block was transferred to a round white teflon plate (diameter 5 cm) and secured with small pins in 87% (v/v) glycerol/water, which results in a clearing of the exocrine tissue. Gentle dissection allowed all islets to be separated from each other. The duodenum, spleen, and stomach were removed, and a glass plate (76 × 40 × 1 mm) was firmly placed on the pancreas. Black and white photographs (Agfaortho 25, Agfa-Gevaert, Leverkusen, Germany) were taken at a fixed magnification in a photomicroscope (Wild M 400, Wild, Heerbrugg, Switzerland), connected to an electronic control unit (MPS 55, Wild, Heerbrugg). Reproducible magnification was assured by photographing a ruler with each pancreas. The film was developed in Rodinal (Agfa, diluted 1 + 20) for 6 min and prints were made on grade-2 paper. Each whole pancreas was covered by approximately 12 photographs (Figure 1). The primary plus secondary magnification was 14 diameters. The photographs were cut into appropriate pieces and placed under a television camera (Bosch Fernseh-Anlagen, Darmstadt, Germany) at a fixed distance. Quantitative image analysis and counts of islets were performed with electronic texture analyzing system (T.A.S. Leitz, Wetzlar GMBH, Germany) which distinguishes differences in image density (gray values). The moveable scanning element is a hexagon yielding a high degree of accuracy in area measurements independent of the configuration of the object. Each islet was registered individually and the area determined. Results are given as islet numbers and area in mm^2. Separate experiments showed that increased pressure on the glass plate did not enlarge the measured area of the islets (data not given). Thus, the area observed can be directly related to islet volume.

Light microscopy. Each whole pancreas was fixed in Bouin's solution with or without perfusion of the pancreas and with or without a final staining with dithizone. Two controls and three SZ-treated mice from each experimental day were processed for light microscopy. Sections were stained with hematoxylin-eosin, coded, and the presence of insulitis was scored independently by three investigators [graded qualitatively from 0 (no insulitis) to 4+ (essentially complete destruction of normal islet architecture)].

RESULTS

Body weight, determined on each experimental day (6–15), fell slowly until day 15, when a significantly lower body weight (89% of that in control mice) was observed. On day 6, the day after the last streptozotozin (SZ) injection, the nonfasting blood glucose was elevated in SZ-injected mice, and it continued to increase throughout the experiment. Fasting blood glucose did not increase until day 9, i.e., 4 days after the last SZ injection (Figure 2).

Perfusion of the normal mouse pancreas with 30 mmol/L D-glucose resulted in a dramatic and pronounced release of insulin (Figure 3). The first phase of release was followed by a second phase of a nearly constant rate of release. On day 6 in the experimental mice, the day after the last injection of streptozotocin, there was a marked reduction in glucose-induced insulin release affecting both the initial and the secondary phase of secretion (Figure 3). The rate of insulin release in response to glucose was further reduced on

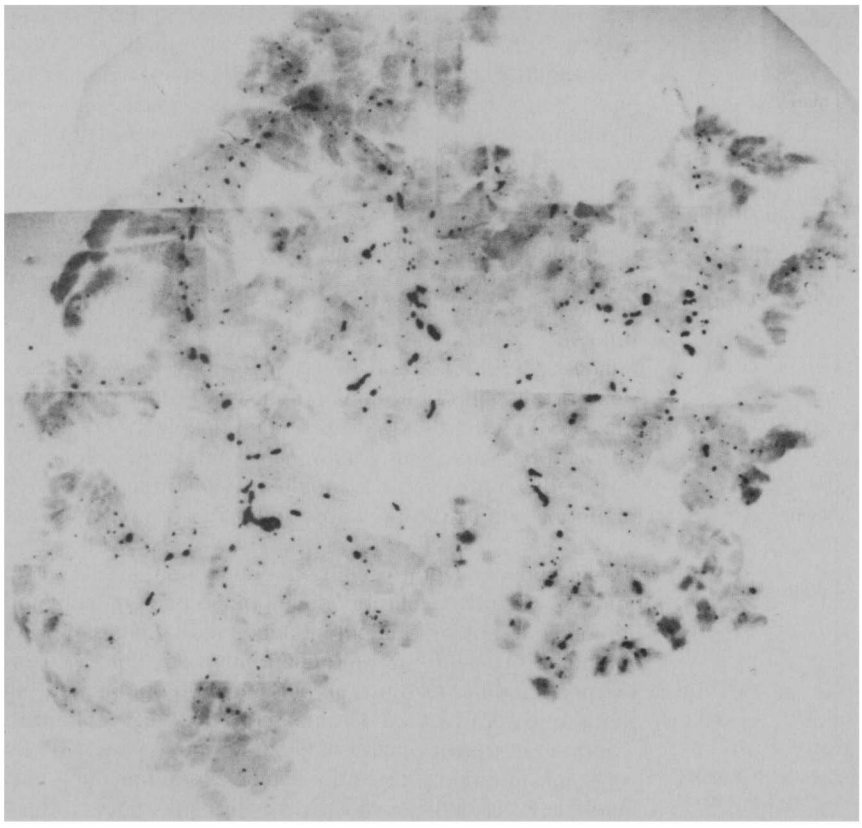


FIGURE 1. Photomontage of a single mouse pancreas stained by perfusion in vitro for 3 min with 1.24 mmol/L dithizone in Krebs-Ringer bicarbonate buffer. The islets remain markedly stained in a red color after perfusion in dithizone-free Krebs-Ringer bicarbonate buffer. Similar photographic preparations were used to determine islet number and area in an electronic texture analyzing system. (×10.)

FIGURE 2. Fasting (16–18 h) or nonfasting blood glucose in C57BL/KsJ-mice given five (arrows) subdiabetogenic doses of streptozotocin. Mean ± SEM for the number of mice shown in parenthesis. Effects of streptozotocin: *P < 0.02, **P < 0.01, ***P < 0.005, ****P < 0.001.

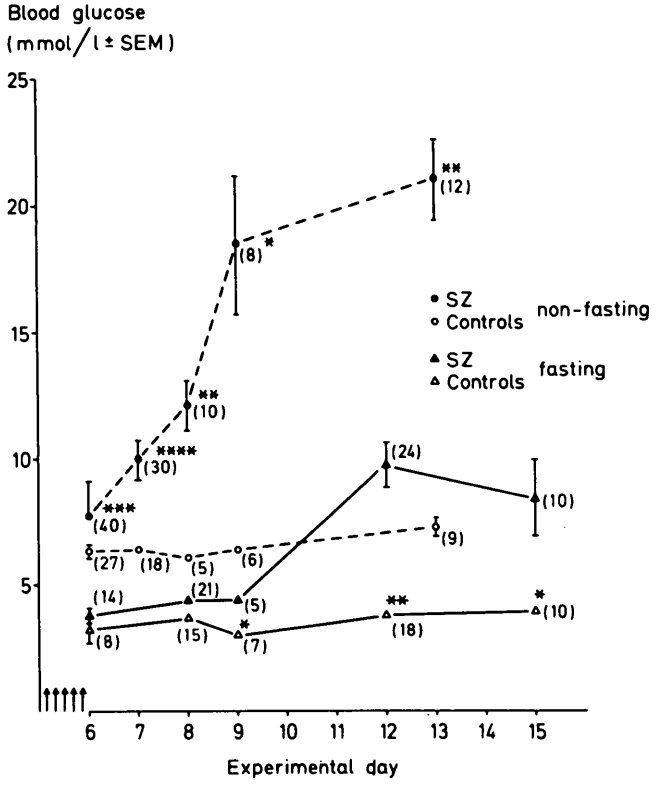
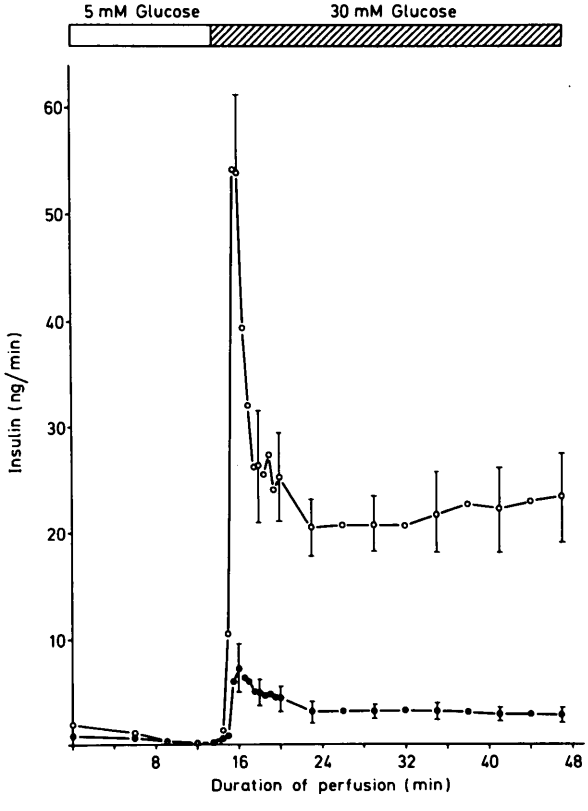


FIGURE 3. Insulin release from the pancreas of C57BL/KsJ-mice perfused on day 6 following 5 days of daily injections without (○——○) or with 40 mg/kg streptozotocin (●——●). The pancreata were first perfused for 12.5 min with 5 mmol/L D-glucose and then with 30 mmol/L D-glucose for another 35 min. Mean values ± SEM for 5 streptozotocin-treated and 12 control mice.



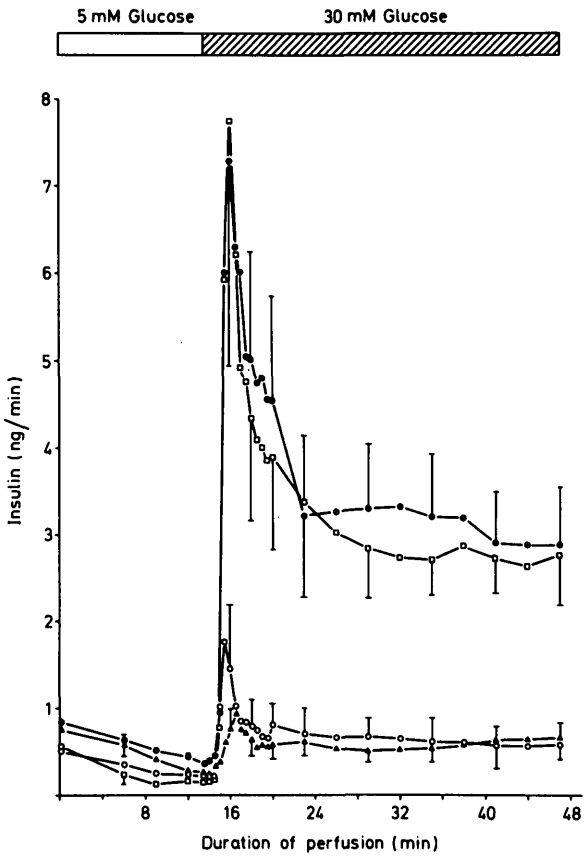


FIGURE 4. Insulin release from the pancreas of C57BL/KsJ-mice perfused following 5 days of daily injections with streptozotocin on day 6 (●——●), day 8 (□——□), day 12 (○——○) and day 15 (▲——▲). Each pancreas was first perfused for 12.5 min with 5 mmol/L D-glucose and then for 35 min with 30 mmol/L D-glucose. Mean values ± SEM for 5–8 mice.

days 8, 12, and 15 (Figures 4 and 5a, and Table 1). The integrated insulin response following SZ declined in a manner compatible with an exponential loss of insulin secretory capacity. Following streptozotocin, a reduction in number of islets became apparent even on day 6 and was more evident on days 12 and 15, when only a few small islets were seen (Figure 5b and Table 1). Islet area, substantially reduced by day 6, seems to fall in a manner suggesting an exponential loss of islet volume (Figure 5c and Table 1).

The development of diabetes in C57BL/KsJ-mice was associated with the presence of inflammatory cell infiltrates in the islets. Control pancreata exhibited no signs of inflammation, whereas streptozotocin-injected mice had round cell infiltration on all experimental days (Table 1). The insulitis seemed to be most pronounced on day 8.

DISCUSSION

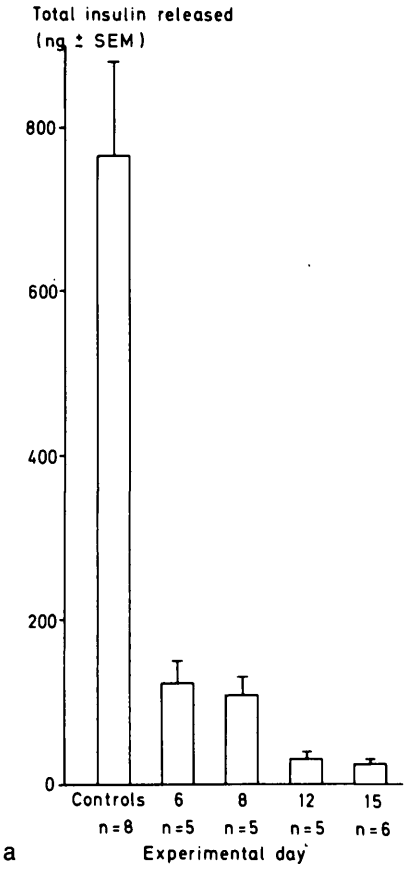
Our results extend previous studies showing that hyperglycemia and inflammatory islet lesions develop in C57BL/KsJ-mice after multiple doses of streptozotocin.[14,15] We documented a markedly reduced insulin secretory capacity coupled with a corresponding decrease in islet area and number as early as day 6, only 1 day after the last dose of streptozotocin. Despite this substantial loss of B-cell function (85% on days 6 and 8), the animals maintained normal fasting blood glucose levels. In contrast, the nonfasting blood glucose concentration was elevated by day 6 and rose progressively thereafter. Fasting blood glucose levels did not rise appreciably until day 12, at which time the animals had less than 4% of a normal islet volume and insulin secretory capacity. Interestingly, insulitis followed the reduction in islet function. By day 6, when only 15% of islet function remained, the insulitis was still increasing, reaching an apparent maximum by day 8. Together, these observations suggest a substantial toxic effect of streptozotocin in reducing islet function. Insulitis follows the initial islet destruction as does a rise in fasting blood glucose once greater than 90% of the islet mass is destroyed.

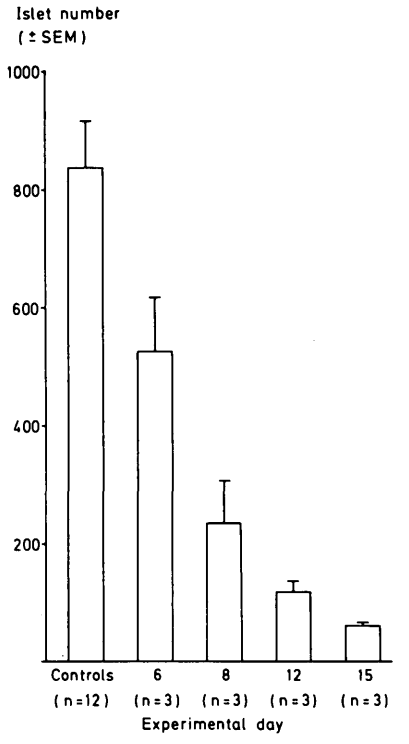
The induction of diabetes after the five injections of streptozotocin was highly reproducible, and in all animals followed a course leading to persistent hyperglycemia. This pathologic process seemed to involve an exponential course with respect to both loss in B-cell function and islet area. However, it cannot be deduced from the present investigation whether the progressive reduction in B-cell function and islet area is the result of an inflammatory reaction in the islets or a B-cell destructive process induced by the drug itself. Previous studies in CD-1 mice[6,16] have shown that 3-O-methyl-D-glucose, a nonmetabolizable glucose analogue given before each streptozotocin injection, provides a partial protection against B-cell destruction and hyperglycemia. It was suggested that the sugar may interfere with a possible direct B-cell cytotoxic effect of streptozotocin. In further experiments[6,16], it was tested whether immunosuppression utilizing a rabbit anti-mouse lymphocyte serum affected the appearance of insulitis and/or hyperglycemia. While anti-lymphocyte serum alone did not prevent the development of diabetes, it was ameliorated by the combined treatment with 3-O-methyl-D-glucose and anti-lymphocyte serum. It was concluded that the low-dose streptozotocin di-

TABLE 1
C57BL/KsJ-mice receiving five low doses of streptozotocin. Changes of several parameters reflecting alterations in islet morphology and function expressed as per cent change from values in control mice or (with insulitis) as a qualitative measure

	Day of the study*						
	6	7	8	9	12	13	15
Insulin secretion	−84%		−86%		−96%		−98%
Islet area	−69%		−88%		−96%		−99%
Islet number	−38%		−72%		−86%		−93%
Nonfasting blood glucose	+27%	+27%	+88%	+194		+190%	
Fasting blood glucose	+14%		+11%	+42%	+111%		+89%
Insulitis	2+		4+		3+		3+

* Day 1 is designated as the day the first dose of streptozotocin was given.





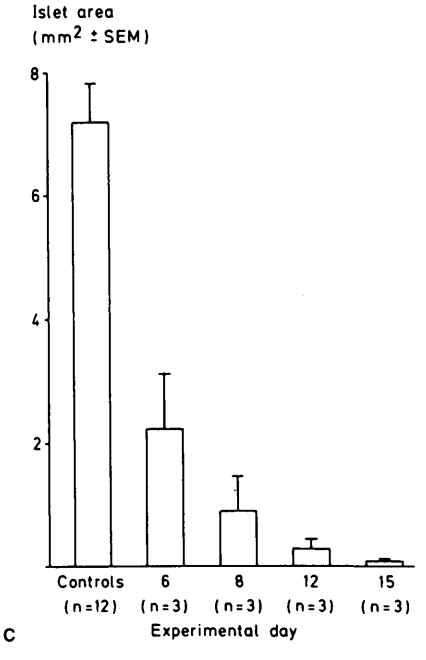


FIGURE 5. Total amount of insulin released during perfusion of the pancreas (a), number of dithizone-stained islets per pancreas (b), and the total area of dithizone-stained islets (c). The analyses were carried out on pancreata from C57BL/KsJ-mice following 5 days of daily injections without (controls) or with 40 mg/kg streptozotocin. Mean values ± SEM for the number of mice shown in the figure.

abetes therefore involves both direct B-cell cytotoxicity and cell-mediated autoimmunity.[6,16] Both mechanisms may not always operate, as hyperglycemia and morphologic evidence of B-cell destruction were induced in rats and in certain strains of mice without a concomitant insulitis.[6,14] On the other hand, diabetes and insulitis did not develop in nude mice unless grafted with a thymus.[17] The approach presented in this study should make it possible for example to study the possible direct effect of B-cell cytotoxic lymphocytes. Passive transfer experiments are attractive in this respect; however, the evidence suggesting successful transfer of diabetogenic lymphocytes[18,19] seems highly ambiguous.[20,21]

By combining quantitative analysis of insulin secretion with careful estimates of islet volume and number, we showed that these parameters were closely related to one another, and perhaps they correlated better with the nonfasting blood glucose level rather than the fasting. The reduction in the amount of insulin released followed the fall in islet area (and volume) more closely than islet number. Most likely, all islets were affected by the destructive process and decreased in volume. Conventional light microscopy combined with immunocytochemical staining also indicated a marked loss of insulin containing cells, while glucagon, somatostatin, and PP-immunoreactive cells remained within the islets.[5] With the continued pathologic process and reduction in islet cell number, many islets eventually disappeared. The decreased capacity to release insulin affected both the initial, rapid increase in insulin release and the second phase of a more constant rate of insulin release. This pattern of insulin release from the isolated mouse pancreas corresponds to recent reports.[11,22] It is interesting to note that the basal rate of insulin release at 5 mmol/L D-glucose was not dramatically changed. The close relationship between insulin secretion and islet volume suggests that the fall in secretory capacity does not reflect an impairment of islet function by the presence of mononuclear cells. Since the infiltrating cells may increase the measured islet volume, the remaining functional islet cells would have to be

secreting insulin, at least normally, or maybe even at an increased efficiency.

Our results strongly suggest that the nonfasting blood glucose concentration demonstrates a greater sensitivity to reduction of insulin secretion than does the fasting glucose value. Perhaps a substantially (85%) reduced secretory capacity still can normalize the fasting blood glucose but fails to have sufficient reserve to produce insulin in response to food intake and the resulting increased carbohydrate load. Yet, on day 6, when the treated animals possessed only 16% of a normal insulin secretory capacity, the nonfasting blood glucose level was only modestly (~20%) elevated. Thus, under normal circumstances, an insulin secretory capacity exceeds the demands placed upon it, and a marked reduction must occur before glucose intolerance can be denoted by any blood glucose measurement. If this observation in mice applies to man, then new approaches must be developed to document impaired islet function before the standard glucose measures become abnormal.

ACKNOWLEDGMENTS

The authors wish to thank Bodil Andreasen and Annegrete Pedersen for excellent technical assistance, and professor Jørn Egeberg and Trine Grainger, Department of Anatomy, University of Copenhagen, for their kind assistance in the preparation of sections for light microscopy.

This study was supported by grants from the National Institutes of Health (AM26190) and Vera and Carl Johan Michaelsens Legat. Vagn Bonnevie-Nielsen had a research fellowship from the University of Copenhagen, and Michael W. Steffes received a Fogarty Senior International Fellowship.

REFERENCES

[1] Block, M. B., Mako, M. S., Steiner, D. F., and Rubenstein, A. H.: Circulating C-peptide immuno-reactivity: studies in normal and diabetic patients. Diabetes *21*:1013–26, 1972.

[2] Rubenstein, A. H., Steiner, D. F., Horwitz, D. L., Mako, M. E., Block, M. B., Starr, J. I., Kuzuya, H., and Melani, F.: Clinical significance of circulating proinsulin and C-peptide. Recent Prog. Horm. Res. *33*:435–75, 1977.

[3] Binder, C., and Faber, O.: Residual beta-cell function and its metabolic consequences. Diabetes *27* (Suppl. 1): 226–29, 1978.

[4] Like, A. A., and Rossini, A. A.: Streptozotocin-induced pancreatic insulitis: new model of diabetes mellitus. Science *193*:415–17, 1976.

[5] Like, A. A., Appel, M. C., Williams, R. M., and Rossini, A. A.: Streptozotocin-induced pancreatic insulitis in mice. Lab. Invest. *38*:470–86, 1978.

[6] Rossini, A. A., Like, A. A., Chick, W. L., Appel, M. C., and Cahill, G. F.: Studies of streptozotocin-induced insulitis and diabetes. Proc. Natl. Acad. Sci. USA *74*:2485–89, 1977.

[7] Gepts, W.: Pathologic anatomy of the pancreas in juvenile diabetes mellitus. Diabetes *14*:619–33, 1965.

[8] Riley, V.: Adaptation of orbital bleeding technique to rapid serial blood studies. Proc. Soc. Exp. Biol. Med. *104*:751–54, 1960.

[9] Grodsky, G. M., and Fanska, R. E.: *In* Methods in Enzymology. Hardman, J. G., and O'Malley, B. H., Eds. New York, Academic Press, 1975, pp. 364–74.

[10] Idahl, L.-Å.: A micro perifusion device for pancreatic islets allowing concomitant recordings of intermediate metabolites and insulin release. Anal. Biochem. *50*:386–98, 1972.

[11] Berglund, O., Frankel, B. J., and Hellman, B.: Development of the insulin secretory defect in genetically diabetic (db/db) mouse. Acta Endocrinol. (Kbh) *87*:543–51, 1978.

[12] Heding, L. G.: A simplified radioimmunoassay method. *In* Labelled Proteins in Tracer Studies. Donato, L., Milhaud, C., and Sirchis, J., Eds. Brussels, Euratom, 1966, pp. 345–50.

[13] Linde, S., Hansen, B., and Lernmark, Å.: Stable iodinated polypeptide hormones prepared by polyacrylamide gel electrophoresis. Anal. Biochem. *107*:165–76, 1980.

[14] Rossini, A. A., Appel, M. C., Williams, R. M., and Like, A. A.: Genetic influence of the streptozotocin-induced insulitis and hyperglycemia. Diabetes *26*:916–20, 1977.

[15] Andersson, A.: Islet implantation normalises hyperglycemia caused by streptozotocin-induced insulitis. Lancet *1*:581–84, 1979.

[16] Rossini, A. A., Williams, R. M., Appel, M. C., and Like, A. A.: Complete protection from low-dose streptozotocin-induced diabetes in mice. Nature *276*:182–84, 1978.

[17] Paik, S.-G., Fleischer, N., and Shin, S.-I.: Induction of diabetes by streptozotocin is mediated by T-cell functions. Diabetes *29* (Suppl. 2):52A, 1980.

[18] Buschard, K., and Rygaard, J.: Passive transfer of streptozotocin-induced diabetes mellitus with spleen cells. Acta Pathol. Microbiol. Scand. [C] *85*:469–72, 1977.

[19] Buschard, K., Madsbad, S., and Rygaard, J.: Passive transfer of diabetes mellitus from man to mouse. Lancet *1*:908–910, 1978.

[20] Beattie, G., Lannom, R., Lipsick, J., Kaplan, N. O., and Osler, A. G.: Streptozocin-induced diabetes in athymic and conventional BALB/c-mice. Diabetes *29*:146–50, 1980.

[21] Neufeld, M., McLaughlin, J., MacLaren, N. K., Rosenbloom, E., and Donnelly, W.: Failure to transfer diabetes mellitus from man to mouse. N. Engl. J. Med. *301*:665, 1979.

[22] Berglund, O.: Different dynamics of insulin secretion in the perfused pancreas of mouse and rat. Acta Endocrinol. (Kbh) *93*:54–60, 1980.