

# Islet loss and alpha cell expansion in type 1 diabetes induced by multiple low-dose streptozotocin administration in mice

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## Abstract

The aim of this study was to investigate the alpha cell population during the development of type 1 diabetes following multiple low-dose streptozotocin administration in mice. For this purpose C57BL/Ks male mice were injected with streptozotocin (40 mg/kg body weight for 5 days). Development of hyperglycemia was monitored over 28 days and a morphometric analysis of islet endocrine cells was performed. A reduction of islet cell area was observed after two injections of streptozotocin. The subsequent decrease of the area throughout the study period averaged 35%. Insulin-positive beta cells gradually disappeared from the identified islets. Hyperglycemia was present from day 7 onwards and in parallel with hyper-

glycemia, insulinitis developed. An analysis of the alpha cell number per islet area revealed a 2- to 3-fold increase in this cell population, with the highest value on day 21. Confocal microscopy analysis of the ICA 512 protein tyrosine phosphatase revealed strong expression in the alpha cells at day 21, suggesting high secretory activity in the diabetic state. It is concluded that multiple low-dose streptozotocin treatment of C57BL/Ks male mice causes the disappearance of a fraction of the islets of Langerhans. In the remaining islet tissue an expansion of alpha cells occurs, reflecting a loss of intrainislet beta cells as well as a regeneration of alpha cells.

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## Introduction

It is well known that the secretion of glucagon is abnormal in human type 1 diabetes patients. The circulating levels of glucagon are normal or elevated in the postabsorptive state, despite the presence of hyperglycemia (Muller *et al.* 1971, Unger & Orci 1981). The patients do not secrete glucagon in response to hypoglycemia (Cryer *et al.* 1994, Hoffman *et al.* 1994) and they have an exaggerated response of glucagon to stimuli such as arginine infusion and a protein meal (Unger & Orci 1981). These phenomena are thought to reflect an intrainislet insulin deficiency, which is thought to desensitize the alpha cells to changes in glucose concentrations (Unger & Orci 1981). In studies of patients with type 1 diabetes there are indications of an increase in alpha cell numbers (Gepts & De Mey 1978, Gepts & Lecompte 1981, Somoza *et al.* 1994), whereas this has not been reported in other investigations (Stefan *et al.* 1982, Rahier *et al.* 1983).

Investigations into the growth potential of the beta cells has elicited considerable interest (Rosenberg *et al.* 1988, Teitelman 1996, Corbett *et al.* 1997). The neoformation of alpha cells was recently described in a study of the non-obese diabetic (NOD) mouse (O'Reilly *et al.* 1997), an experimental model of type 1 autoimmune diabetes known to display hyperglucagonemia similar to that found in human type 1 diabetes (Ohneda *et al.* 1984). The alpha

cells were thought to be derived both from progenitor cells present in ductal areas and from replication of islet alpha cells. An appearance of alpha cells is of special interest since the ontogeny of the beta cell may evolve via expression of genes for other islet hormones, especially glucagon, before the beta cell phenotype is attained (Teitelman 1996).

The aim of the present study was to examine changes of the pancreatic islets with special attention to an appearance of alpha cells following administration of streptozotocin to C57BL/Ks mice. Streptozotocin is a beta cell toxin (Rakieten *et al.* 1963, Wilson & LeDoux 1989), which when given in low doses to certain strains of mice induces insulin deficiency and hyperglycemia, typical of type 1 diabetes, through a process dependent upon both the direct toxicity of the drug and immune effector mechanisms (Like & Rossini 1976, Kolb 1987). The disease occurs within 2–3 weeks in a reproducible manner, allowing detailed studies of the islets. Investigations of the alpha cells in this disorder have not been described previously.

## Materials and Methods

### *Animals and treatment*

Inbred 12- to 16-week-old C57BL/Ks male mice (Biomedical Center, Uppsala, Sweden), originally obtained

from the Jackson Laboratory (Bar Harbor, ME, USA) were used. The animals had free access to tap water and pelleted food (R34; AnaLyzen, Lidköping, Sweden). The mice were treated with i.p. injections of either saline (0.2 ml) or streptozotocin (40 mg/kg body weight) dissolved in saline for 5 consecutive days. Some animals were killed, by cervical dislocation, before any injections (day 0).

Blood glucose determinations (ExacTech blood glucose meter; Baxter Travenol, Deerfield, IL, USA) were performed on blood samples taken from the tail tip on day 0 before any injection, and subsequently 3, 7, 10, 14, 21 and 28 days after the first injection. On these days groups of five mice were killed for morphological examination of the pancreatic glands.

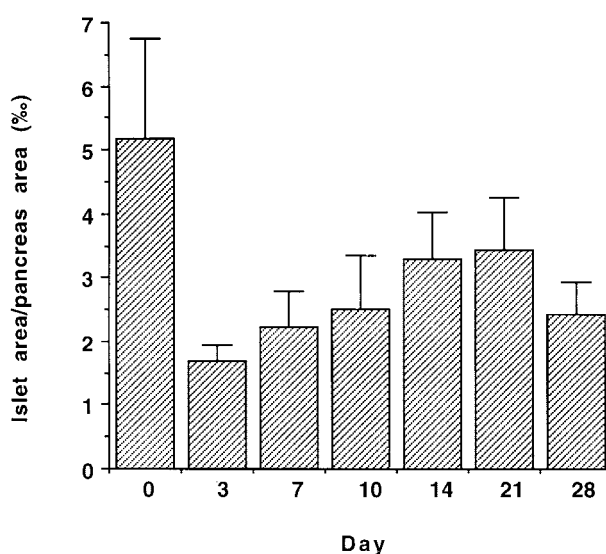
### Morphological examinations

After cervical dislocation, pancreatic glands were removed from the mice, a part fixed in 10% formalin solution, and the other part rapidly frozen in liquid nitrogen for confocal microscopy. For light microscopy the fixed glands were embedded in paraffin and sections, 5 µm thick, were cut and stained using the peroxidase–anti-peroxidase-technique to demonstrate alpha cells (rabbit anti-porcine glucagon serum (1:200); Dakopatts AB, Älvsjö, Sweden), beta cells (guinea pig anti-porcine insulin (1:100); Dakopatts) and pancreatic polypeptide (PP) cells (a rabbit polyclonal anti-human pancreatic polypeptide serum (1:600); Dakopatts).

Pancreatic islet mononuclear cell infiltration was ranked according to an arbitrary scale (0–3). Rank 0 denotes no lymphocytic infiltration; rank 1 denotes <10% of the islets infiltrated; rank 2 denotes 10–50% of the islets infiltrated; rank 3 denotes >50% of the islets infiltrated. For each animal a mean score was calculated = total score for all islets examined/total number of islets examined. The pancreatic sections were evaluated with the examiner being unaware of the origin of the sections.

The distribution of expression of ICA 512, a marker of neurosecretory granules and a putative islet autoantigen, considered to be present on all islet endocrine cells (Solimena *et al.* 1996), was examined by confocal microscopy on day 21. For this purpose double immunohistochemical staining was performed on 6 µm thick sections. Rabbit antiserum (89–59), diluted 1:400, against the cytoplasmic domain of the ICA 512 protein (a generous gift from Bayer, Elkhart, IN, USA) and monoclonal anti-glucagon (mouse ascites clone K796B10, 1:1000; Sigma Chemical Co., St Louis, MO, USA) were used.

The number of alpha cells per islet area was determined by confocal microscopy (Zeiss LSM 410 Invert Laser Scan Microscope; Carl Zeiss Jena GmbH, Jena, Germany) at different time points after saline or streptozotocin injection. This was performed by measurements of >2000 µm<sup>2</sup> islet area selected from >20 islets/pancreas. For each pancreas four to seven sections (>50 µm apart)



**Figure 1** Islet area in relation to whole pancreatic area in light microscope sections on different days after starting streptozotocin treatment. Values are means ± S.E.M. for five animals, except for day 28 when four animals were examined.

were examined. In islets with lymphocytic infiltrates, the islet area did not include peri-insular lymphocytes located outside the endocrine cell mass. The alpha cell numerical density per animal was defined as the total alpha cell number/total islet area. The alpha cell number per whole pancreas area was measured on the same sections, as well as the total islet area per total pancreatic area.

The islet PP cell density was counted in light microscopical sections, at each time point, in two or three pancreatic sections, taken >50 µm apart from four or five animals. For each animal a mean score = total PP-positive cell number/total number of islets examined, was determined.

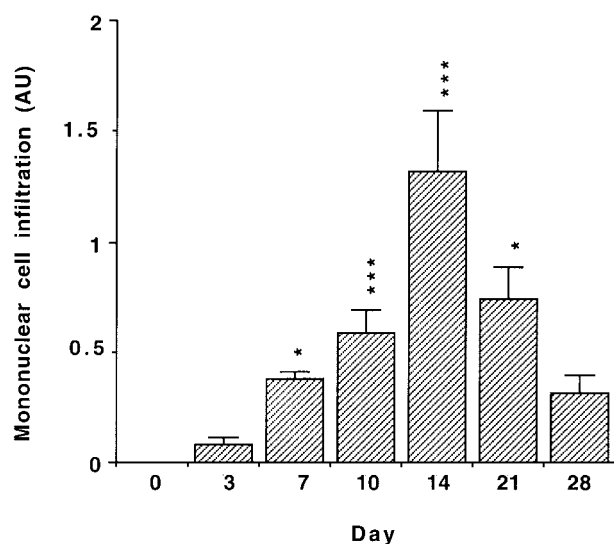
### Statistical analysis

Data were expressed as means ± S.E.M. and groups of data were compared using Student's unpaired *t*-test.

### Results

Mice injected with multiple low doses of streptozotocin developed a gradually increasing hyperglycemia from day 7 onwards (data not shown). The saline-treated control mice remained normoglycemic throughout the observation period.

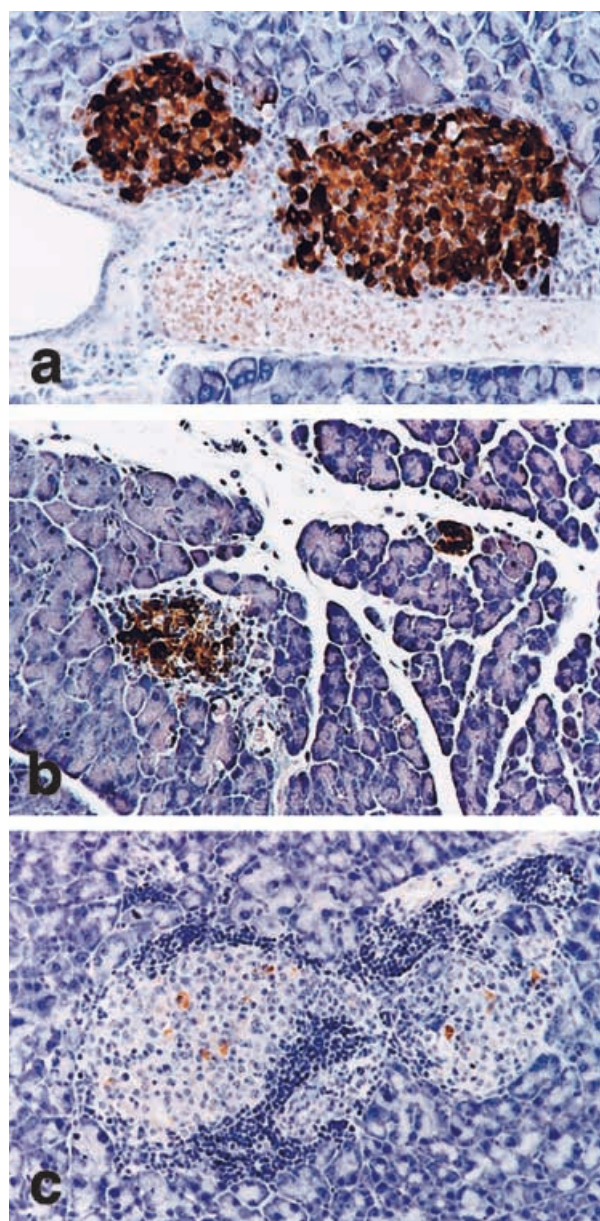
The islet area in relation to the total pancreas area decreased after streptozotocin administration (Fig. 1). When pooling the data from different days after day 0 and comparing saline and streptozotocin-injected mice, the islet area had decreased by about 35% ( $P=0.0155$ ) in the streptozotocin group.



**Figure 2** Magnitude of mononuclear cell infiltration in the pancreatic islets on different days after starting streptozotocin treatment. The cell infiltration was scored according to an arbitrary unit (AU) scale described in the Materials and Methods section. Values are means  $\pm$  S.E.M. for five observations. \* $P < 0.05$ , \*\*\* $P < 0.001$  compared with day 0, using an unpaired Student's *t*-test. On day 0 all five animals were scored as 0.

On day 0 before any drug had been administered, no signs of mononuclear cell infiltration were observed (Figs 2 and 3a (day 0, insulin)). In the control group this was the case during the whole 28 day period (not shown). After streptozotocin treatment, islet cell infiltration was seen after 7 days and increased further until day 14 (Figs 2 and 3b and c (day 7 and 14, insulin)). Thereafter, the infiltration started to decline and on day 28 the infiltration was similar in magnitude to that on day 7 (Fig. 2). The staining of islet beta cells decreased after commencement of the streptozotocin injections. On day 7 some degranulation of the beta cells was seen which was accompanied by immune cell accumulation around the islets (Fig. 3b (day 7, insulin)), and on day 14 the insulin-positive cells were scarce (Fig. 3c (day 14, insulin)). However, even on day 28, when the islet tissue was much reduced, a few positive beta cells still remained (not shown).

The alpha cells had a typical peripheral location in the islets seen on day 0 (Fig. 4a). On day 14, in parallel with marked insulinitis, most alpha cells remained in the periphery, but some alpha cells had become more centrally orientated within the islet (Fig. 4b). When examined on day 21, centrally located alpha cells were frequent and the number of alpha cells per islet appeared increased (Fig. 4c). At this stage, glucagon-positive cells were also occasionally found in the pancreatic duct epithelium (Fig. 4d). A morphometric analysis of the alpha cell number per islet area revealed a 2- to 3-fold increase in this cell population, with the highest value on day 21 (Fig. 5a). When the alpha

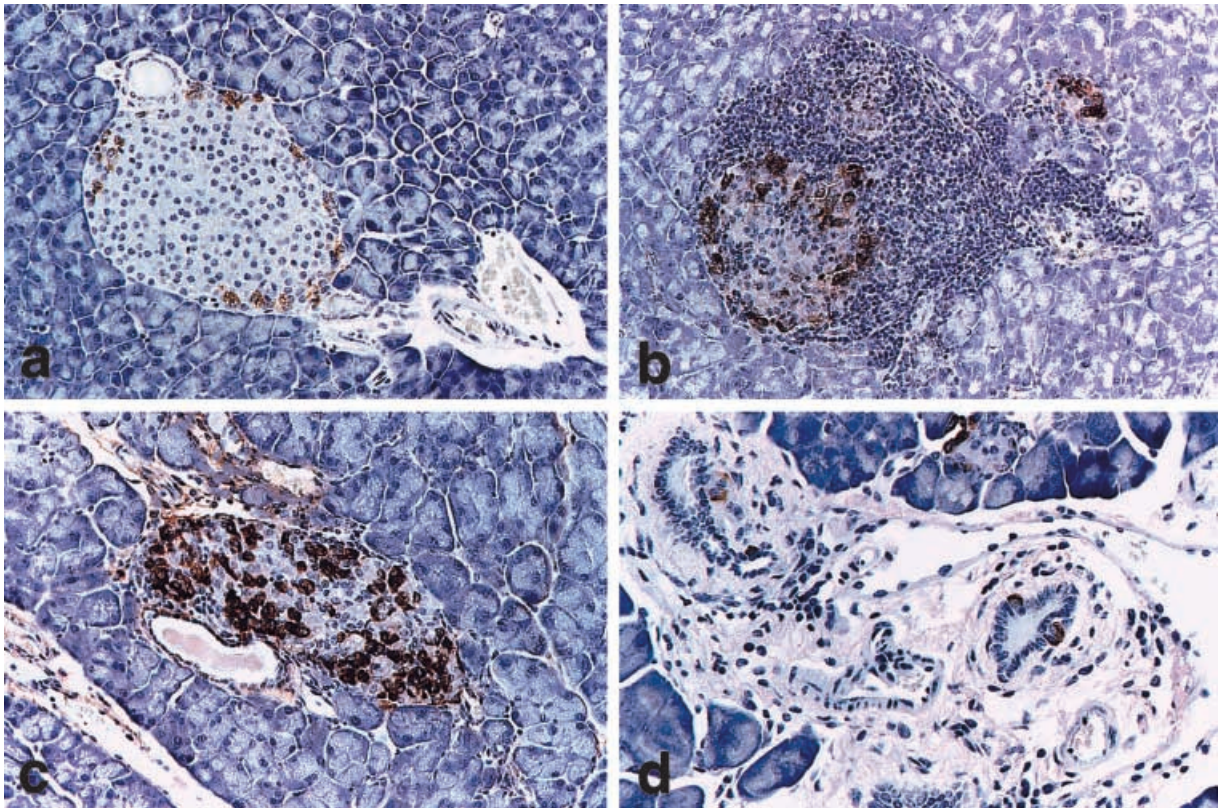


**Figure 3** Light microscopy view of pancreatic islets in sections immunocytochemically stained for insulin, (a) before any treatment, (b) 7 days, and (c) 14 days after starting injections of streptozotocin. A marked reduction of insulin-positive cells and infiltration of immune cells in the islet area can be seen on day 14. Magnification  $\times 312$ .

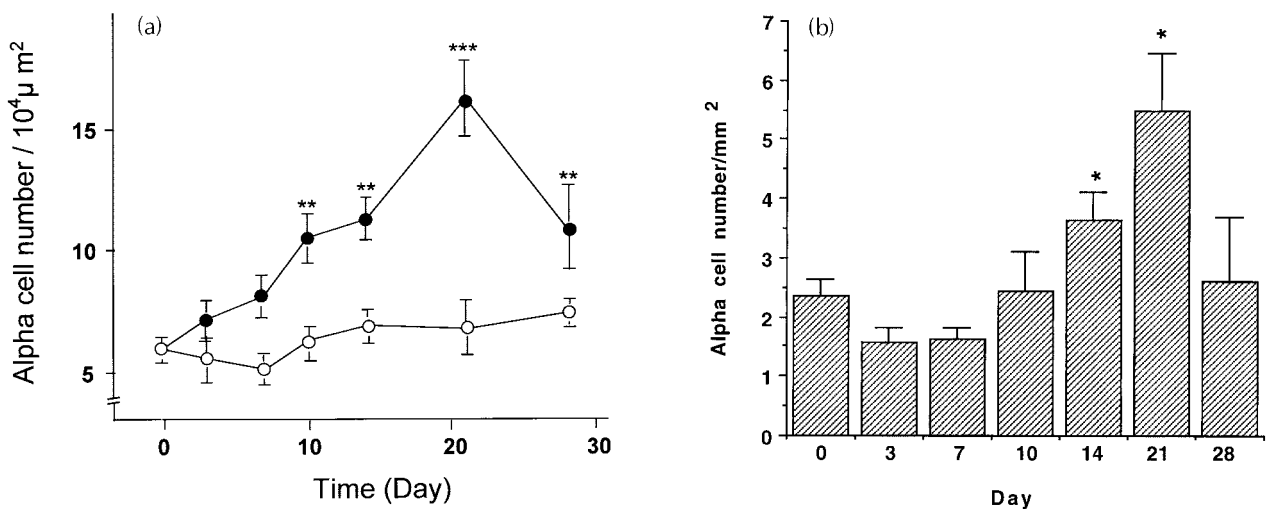
cell number was expressed per whole pancreatic area there was a significant increase compared with day 0 on days 14 and 21 (Fig. 5b).

The PP cell number per islet remained low and unchanged (approximately two to four per islet section) after streptozotocin administration, over the 28 days (PP cells/islet section: day 0,  $3.4 \pm 0.60$ ; day 3,  $1.8 \pm 0.34$ ;

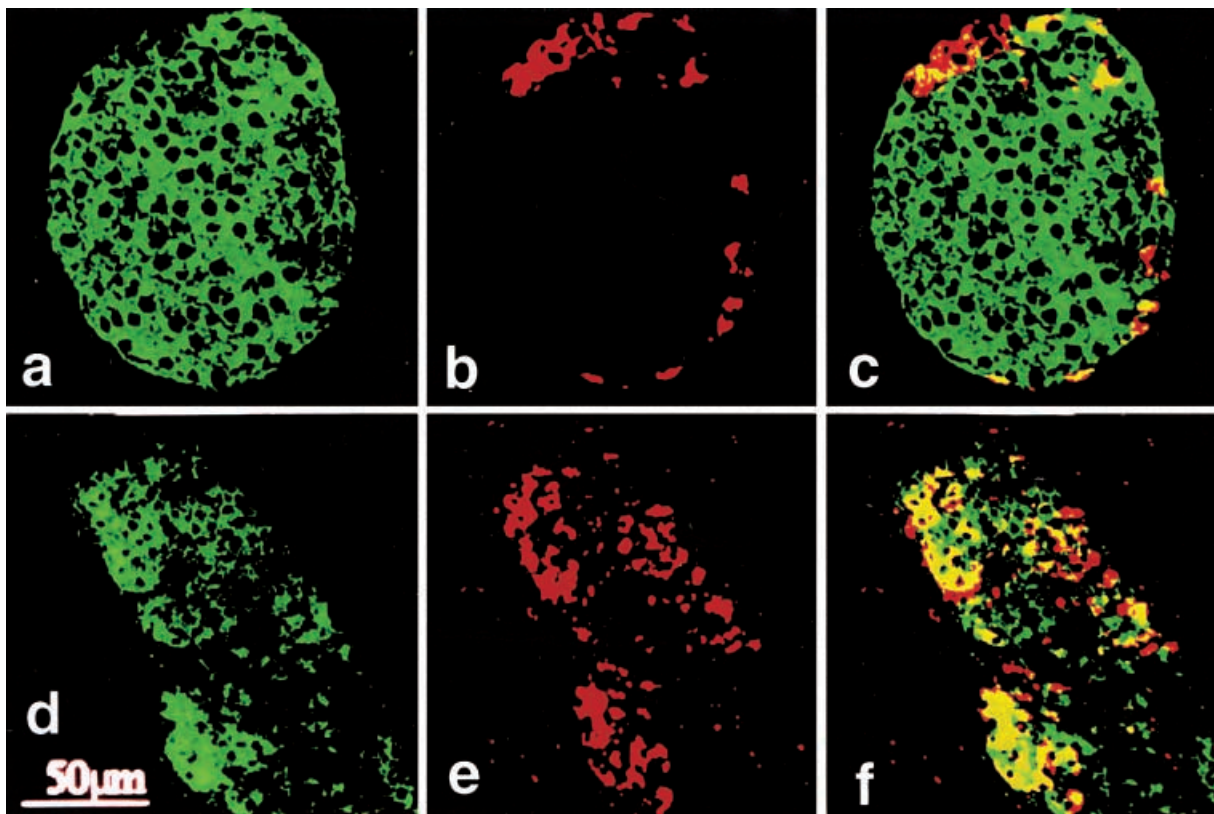




**Figure 4** Light microscopy view of pancreatic sections immunocytochemically stained for glucagon, (a) before any treatment, (b) 14 days, and (c, d) 21 days after starting injections of streptozotocin. Especially on day 21, an increased number of glucagon-positive cells can be seen within the islets (c) as well as occasional glucagon-positive cells in the pancreatic duct epithelium (d). Magnification  $\times 312$  (a–c) and  $\times 468$  (d).



**Figure 5** (a) Morphometric analysis of alpha cell number per islet area on different days after starting streptozotocin (●) or saline (○) treatment.  $***P < 0.01$  and  $**P < 0.01$  vs saline-treated mice, using an unpaired Student's *t*-test. (b) Alpha cell number per whole pancreas area.  $*P < 0.05$  vs day 0, using an unpaired Student's *t*-test. Values are means  $\pm$  S.E.M. for five animals, except for the streptozotocin group on day 28, when four animals were examined, and for the saline group on days 7 and 10 where two animals were examined.



**Figure 6** Confocal microscopy of pancreatic islets on day 0 (a–c) and 21 days after starting injections of streptozotocin (d–f). Cells expressing the ICA 512 antigen are green, glucagon-positive cells are red, and glucagon-positive cells expressing ICA 512 are stained yellow.

day 7,  $2.4 \pm 0.55$ ; day 10,  $2.0 \pm 0.41$ ; day 14,  $2.4 \pm 0.51$ ; day 21,  $2.25 \pm 0.48$ ; day 28,  $4.0 \pm 0.35$ ). As compared with day 0 there was a trend towards a decline on day 3, but this difference did not attain statistical significance ( $P=0.053$ ). In animals treated with saline injections the PP cell number/islet section (three to four; data not shown) remained similar throughout the 28 days.

Using confocal microscopy it was found that the ICA 512 antigen was present over the whole islet area, presumably in all islet endocrine cells, on day 0 (Fig. 6a–c). Following streptozotocin injections and the altered localization of the alpha cells, the presence of the ICA 512 antigen seemed to change in a pattern similar to the alpha cell distribution (Fig. 6d–f).

## Discussion

In the present study, we noted a rapid loss of islets following the administration of streptozotocin and after two doses, i.e. on day 3, the islet area per pancreas area was reduced by 70%. No further reduction took place although additional streptozotocin was given on day 3, 4 and 5. This

is in line with previous investigations and strengthens the view that an important component of beta cell destruction in the low-dose streptozotocin model is toxicity to the beta cells which precedes insulinitis (Bonnievie-Nielsen *et al.* 1981, Sandler 1984). In our study, the islet area rather increased to an average of about 50% of the original at the end of the study on day 28. The latter finding varies from the previous report by Bonnievie-Nielsen *et al.* who found a reduction of the islet area down to 31% on day 6, and a further decline to 1% of the original area on day 14 (Bonnievie-Nielsen *et al.* 1981). The same treatment protocol and strain of mice (C57BL/Ks) were used as in our study. In their study the islet area was estimated by a procedure of *in vivo* infusion of dithizone to stain the islets, followed by immersion of the pancreatic tissue in 87% glycerol/water. This was stated to result in clearing of the exocrine pancreas and to allow freeing of all islets. The islets were detected individually and the area estimated by quantitative image analysis. In our study, the islets were identified on the basis of glucagon staining. The borders of the islets were identified and the individual islet area determined by image analysis. We observed a reduction in islet area when examined on day 3, but no additional



reduction. Possibly, the technique of Bonnevie-Nielsen *et al.* might have difficulties in freeing islets infiltrated with lymphocytes. Alternatively, the dithizone staining of insulin-deficient islets may be weak, giving falsely low estimates of islet area, although dithizone, via chelation of zinc, is supposed to react with both insulin and glucagon (Logothetopoulos *et al.* 1964).

The alpha cell population changed in a pattern opposite to that of beta cells, and a 2- to 3-fold increase relative to the islet area was observed, whereas similar change did not seem to be the case for the PP cell population. A number of factors might operate in parallel to explain the apparent alpha cell increase during the 28 day follow-up in the present study. First, due to a preferential beta cell loss the relative fraction of alpha cells per islet will increase. This may also lead to a more central location of alpha cells within the islets, as centrally located beta cells are dying and disappear. Secondly, the multiple low-dose streptozotocin administration could promote a selective survival of a subpopulation of relatively toxin resistant islets, i.e. alpha cell-rich islets. Thirdly, a neof ormation of alpha cells may have taken place as has been reported in the NOD mouse pancreas (O'Reilly *et al.* 1997). We observed alpha cells in pancreatic duct epithelium after the streptozotocin administration. It is likely that this represents new alpha cells, but we do not know if these alpha cells will form new islets or migrate into existing islets. Islet cell neogenesis is of particular interest since islet cell stem cells have been found to express multiple islet hormones and the existence of a PDX-1/glucagon-positive embryonic stem cell capable of developing into insulin-producing cell has been suggested (O'Reilly *et al.* 1997). ICA 512 protein tyrosine phosphatase is an integral membrane protein localized to secretory granules of various neuroendocrine cells including all islet cells (Solimena *et al.* 1996). On day 21 after the streptozotocin injections it was found that alpha cells were intensively positive for the protein. This suggests that these cells had an increased secretory activity. This is in accordance with the view that lack of insulin will lead to a decreased suppression of glucagon secretion from the alpha cells and subsequent hyperglucagonemia (Unger & Orci 1990).

The results of the present study demonstrate that an expansion of the alpha cell mass accompanies the development of diabetes in C57BL/Ks mice subjected to multiple low-dose streptozotocin treatment. Islet cell regeneration represents a long-term goal in type 1 diabetes. Perhaps the current experimental model provides a tool to explore a putative role of alpha cells in formation of insulin-producing beta cells.

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## References

- Bonnevie-Nielsen V, Steffes MW & Lermmark Å 1981 A major loss in islet mass and B-cell function precedes hyperglycemia in mice given multiple low doses of streptozotocin. *Diabetes* **30** 424–429.
- Corbett J, Serup P, Bonner-Weir S & Nielsen JH 1997 Beta-cell ontogeny: growth and death. *Diabetologia* **40** B27–B32.
- Cryer PE, Fisher JN & Shamoon H 1994 Hypoglycemia. *Diabetes Care* **17** 734–755.
- Gepts W & De Mey J 1978 Islet cell survival determined by morphology. An immunocytochemical study of the islet of Langerhans in juvenile diabetes mellitus. *Diabetes* **27** 251–261.
- Gepts W & Lecompte PM 1981 The pancreatic islets in diabetes. *American Journal of Medicine* **70** 105–115.
- Hoffman RP, Singer-Granick C, Drash AL & Becker DJ 1994 Abnormal alpha cell hypoglycemic recognition in children with insulin dependent diabetes mellitus (IDDM). *Journal of Pediatric Endocrinology* **7** 225–234.
- Kolb H 1987 Mouse models of insulin dependent diabetes: low dose streptozotocin-induced diabetes and nonobese diabetic (NOD) mice. *Diabetes/Metabolism Reviews* **3** 751–778.
- Like AA & Rossini AA 1976 Streptozotocin-induced pancreatic insulinitis: a new model of diabetes mellitus. *Science* **193** 415–417.
- Logothetopoulos J, Kaneko M, Wrenshall GA & Best CH 1964 Zinc, granulation and extractable insulin following hyperglycemia of prolonged treatment with insulin. In *The Structure and Metabolism of the Pancreatic Islets*. Wenner-Gren Center International Symposium Series, vol 3, pp 333–347. Eds SE Brolin, B Hellman & H Knutson. Oxford, London, Edinburgh, New York, Paris, Frankfurt: Pergamon Press.
- Muller WA, Faloona GR & Unger RH 1971 The effect of alanine on glucagon secretion. *Journal of Clinical Investigation* **50** 1992–1999.
- Ohneda A, Kobayashi T, Nihei J, Tochino Y, Kanaya H & Makino 1984 Insulin and glucagon in spontaneously diabetic non-obese mice. *Diabetologia* **27** 460–463.
- O'Reilly LA, Gu D, Sarvetnick N, Edlund H, Philips JM, Fulford T & Cooke A 1997  $\alpha$ -Cell neogenesis in an animal model of IDDM. *Diabetes* **46** 599–606.
- Rahier J, Goebbels RM & Henquin JC 1983 Cellular composition of the human diabetic pancreas. *Diabetologia* **24** 366–371.
- Rakieten N, Rakieten ML & Nadkarni MV 1963 Studies on the diabetogenic action of streptozotocin. *Cancer Chemotherapy Reports* **29** 91–98.
- Rosenberg L, Duguid WP, Brown RA & Vinik AI 1988 Induction of nesidioblastosis will reverse diabetes in Syrian golden hamster. *Diabetes* **37** 334–341.
- Sandler S 1984 Protection by dimethyl urea against hyperglycaemia, but not insulinitis, in low dose streptozotocin-induced diabetes in the mouse. *Diabetologia* **26** 386–388.
- Solimena M, Dirks R Jr, Hermel JM, Pleasic-Williams S, Shapiro JA, Caron L & Rabin DU 1996 ICA 512, an autoantigen of type 1 diabetes, is an intrinsic membrane protein of neurosecretory granules. *EMBO Journal* **15** 2102–2114.
- Somoza N, Vargas F, Roura-Mir C, Vives-Pi M, Fernandez-Figueras MT, Ariza A, Gomis R, Bragado R, Marti M, Jaraquemada D & Pujol-Burel R 1994 Pancreas in recent onset insulin-dependent diabetes mellitus. Changes in HLA, adhesion molecules and

- autoantigens, restricted T cell receptor V $\beta$  usage and cytokine profile. *Journal of Immunology* **153** 1360–1377.
- Stefan Y, Orci L, Malaisse-Lagae F, Perrelet A, Patel A & Unger RH 1982 Quantitation of endocrine cell content in the pancreas of nondiabetic and diabetic humans. *Diabetes* **31** 694–700.
- Teitelman G 1996 Induction of beta-cell neogenesis by islet injury. *Diabetes/Metabolism Reviews* **12** 91–102.
- Unger RH & Orci L 1981 Glucagon and the A cell: physiology and pathophysiology. *New England Journal of Medicine* **304** 1518–1524.
- Unger RH & Orci L 1990 Glucagon. In *Diabetes Mellitus. Theory and Practice*, pp 104–120, edn 4. Eds H Rifkin & D Porte Jr. New York, Amsterdam, London: Elsevier.
- Wilson GL & LeDoux SP 1989 The role of chemicals in the etiology of diabetes mellitus. *Toxicology and Pathology* **17** 357–363.

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