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Vascular permeability of pancreatic islets after administration of streptozotocin*

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Summary. In the present study we have investigated whether the pancreatic B-cell toxic agent streptozotocin (SZ) induces increased vascular permeability as an indicator of lesions in the pancreatic islets. SZ was given either in multiple low doses, providing an animal model for Type I diabetes mellitus with signs of autoimmunity, or by a single diabetogenic dose to C57BL/KsJ mice. The vascular reactions were detected by administration of Monastral blue B and the pancreatic islets were visualized by a freeze-thawing technique which made it possible to count the number of stained and unstained islets. This proved to be a rapid and sensitive technique for detection of early lesions within the islets after administration of SZ. It was found that the islets showed an increased vascular staining before the animals had become diabetic by either mode of SZ treatment and also before signs of pancreatic insulitis were found after the multiple low-dose injections of SZ. It is suggested that both types of SZ administration induce a B-cytotoxic reaction, essential for the development of hyperglycaemia. This leads to an activation of cells dealing with the disposal of cell debris, a process which probably also involves the release of substances mediating increased vascular permeability.

Key words: Pancreatic islets – Streptozotocin – Insulitis – Monastral blue B – Vascular labelling

Introduction

A common finding in the pancreas at the onset of human Type I diabetes mellitus is an inflammatory lesion with an infiltration of mononuclear cells into the islets of Langerhans (Gepts 1965). It has been assumed that this

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reflects a destructive process affecting the majority of the pancreatic B-cells and resulting in insulin-dependent diabetes mellitus. One animal model for human Type I diabetes mellitus is the multiple low-dose streptozotocin (SZ) induced diabetes in the mouse. This syndrome is characterized by a gradually developing hyperglycaemia and pancreatic insulitis following repeated administration of subdiabetogenic doses of SZ to certain mouse strains (Like and Rossini 1976). The insulitis reaction seems to depend on intact T-lymphocyte function (Paik et al. 1980; Paik et al. 1982; Nakamura et al. 1984) and recent data have also suggested that cytotoxic T-lymphocytes specific for antigens expressed by insulin-producing cells are produced (McEvoy et al. 1984). However, other data have indicated that the insulitis per se in the multiple-SZ treated mice is not sufficient to cause hyperglycaemia (Sandler 1984) and that the insulitis may be secondary to islet cell degeneration due to the B-cytotoxic actions of SZ (Bonnevie-Nielsen et al. 1981).

In the present study we have investigated to what extent the multiple SZ induced insulitis is associated with vascular lesions in the pancreatic islets, and the temporal relationship of such structural changes to the development of hyperglycaemia. Corresponding experiments were performed after a single diabetogenic dose of SZ, where B-cell destruction is not usually accompanied by an insulitis. The inflammatory reactions in the pancreatic islets were monitored by vascular staining with Monastral blue B (Joris et al. 1982), a colloidal pigment which, after intravenous injection, is trapped in the wall of blood vessels with increased permeability, particularly in the postcapillary venules. To distinguish the Monastral blue B staining of the islet vasculature from that of the acinar pancreas the glands were treated for visualization of the islets using a freeze-thawing technique (Jansson and Hellerström 1981) and the number of pigment stained and unstained islets was counted.

Materials and methods

Adult, male inbred C57BL/KsJ mice originally obtained from the Jackson Laboratories (Bar Harbor, Maine, USA) aged 12–16 weeks were used. The animals were allowed free access to tap water and pelleted food (Ewos-Anticimex, Type R3; Ewos, Södertälje, Sweden) throughout the experimental period. Streptozotocin (lot 60,273-5U9889) was kindly supplied by Dr. W.E. Dulin (Upjohn Company, Kalamazoo, Michigan, USA). The drug was dissolved in a 10 mM citric acid buffer (pH 4.5) not more than 2 min before use and then administered via either a single intravenous dose of 160 mg/kg body-weight or by daily intraperitoneal injections of 40 mg/kg body-weight for up to five consecutive days. The control animals were correspondingly injected in parallel with 0.2 ml citric acid buffer only.

A first blood sample was taken by retroorbital sinus puncture for determination of the serum glucose concentration, using an automated glucose oxidase method (Glucose Analyzer 2, Beckman Instruments, Fullerton, California, USA) before SZ treatment. A second sample was taken after different time intervals (single dose animals: 30 min, 18 h, 24 h, 3 days and 8 days; multiple-injected animals: 2, 3, 7, 10 and 14 days after the first injection) following the administration of SZ. After the second blood sample the mice received a single intravenous injection of 0.1 ml Monastral blue B suspension (3% (w/v) phthalocyanine blue pigment dissolved in a solution containing 0.85% (w/v) sodium chloride; Sigma Chemicals, St. Louis, Missouri, USA) and they were killed by cervical dislocation 60 min later. The pancreatic glands were quickly dissected free from surrounding tissues and approximately half of each gland was fixed in Bouin's solution and embedded in paraffin.

Pancreatic sections (7 μm thick) were stained with haematoxylin-eosin and examined for inflammatory reactions within the islets. The examiner was unaware of the origin of the sections (Sandler and Andersson 1982). Islet histology was arbitrarily ranked according to four classes as previously illustrated (Sandler and Andersson 1985): Class A denotes normal islet morphology, class B a low degree of mononuclear cell infiltration especially in the peri-insular area, class C a heavy infiltration with mononuclear cells into a large number of islets and class D only a few residual islets displaying cellular disarray and pyknotic nuclei.

The other half of the dissected pancreas was treated for visualization of the pancreatic islets with a freeze-thawing technique previously described in detail (Jansson and Hellerström 1981). For this purpose the glands were cut in small pieces, each weighing approximately 10–15 mg, and placed between two object slides. The preparations were frozen at −20° C and then allowed to thaw at room temperature. This treatment made the exocrine parts of the glands transparent and the islets could be clearly seen in dark field illumination under a stereo microscope (Wild Heerbrug Ltd., Heerbrug, Switzerland). The Monastral blue B pigment entrapped within the vessel walls could, however, be visualized better in bright field illumination. The practical procedure was therefore first to localize the pigment in bright field illumination and then confirm its localization by changing the illumination of the microscope to dark field. The total number of islets was counted in each preparation and the fraction of islets containing blue pigment was calculated. Only islets with diameters exceeding 50 μm were counted due to the difficulties of distinguishing smaller islets from connective tissue septa.

All values are expressed as means ± SEM. Groups of data were compared using the unpaired Student's two-tailed t-test.

Results

Distribution of the monastral blue B pigment. On dissection of the pancreas, 60 min after the pigment injection, it was found that the livers and spleens of the animals were distinctly blue, probably depending on an active phagocytosis of the colloidal pigment by cells in these organs. Other parenchymatous organs such as the lungs and kidneys remained unstained as previously reported (Joris et al. 1982). In those islets which showed Monastral blue B staining, pigment was preferentially accumulated in the islet periphery. In the exocrine pancreas deposits of pigment were occasionally found in the connective tissue surrounding the pancreatic ducts, irrespective of whether the animals had been given SZ or not. These areas were easily distinguished from the stained islets.

Effects of a single, high dose of SZ. Injection of a high dose of SZ caused a pronounced hypoglycaemia after 18 h followed by a manifest hyperglycaemic state after three days (Table 1). By 24 h after the SZ-injection the number of islets visible in the frozen-thawed pancreatic preparations was reduced and after three days the islet number had decreased to about 25% of that in the control animals. The decrease in the number of visible islets following SZ administration was accompanied by an increased islet staining with the Monastral blue B pigment (Fig. 1). The vascular staining was, however, not increased until 24 h after the SZ-injection, whereas the hypoglycaemic phase following the SZ-injection was observed earlier, i.e. after 18 h. Evaluation of the pancreatic islet histology revealed significant structural alterations in the SZ-treated mice after 18 h (Table 2). After this time

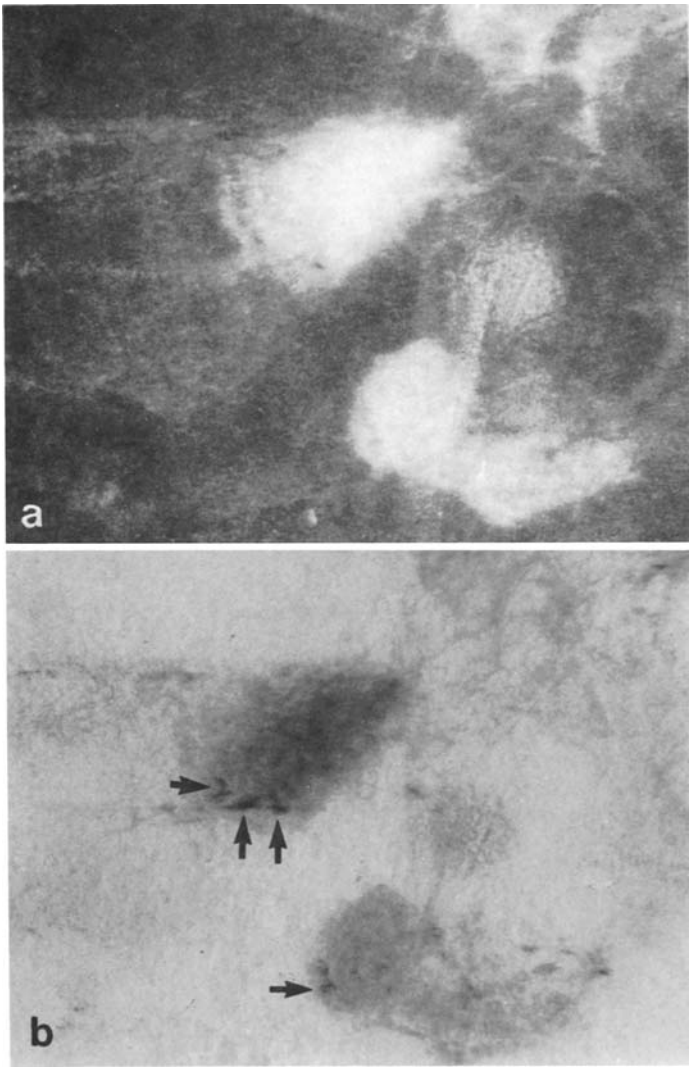

Fig. 1. Stereo-microscopic view of two islets seen in dark field illumination **A** in the frozen-thawed preparation of a pancreatic gland 24 h after injection of a high dose of streptozotocin. Uptake of Monastral blue B pigment within the islets is confirmed by changing to bright field illumination (*arrows* pointing on dark spots; **B**). Magnification 530 ×

interval, most pancreatic glands contained only few and partly disintegrated islets (class D).

Effects of multiple low doses of SZ. The multiple low-dose treatment with SZ induced a slowly developing hyperglycaemia, which did not become manifest until a week after the first of the five consecutive SZ-injections

Table 1. Serum glucose concentrations of male C57BL/KsJ mice, total number of islets observed in approximately half the pancreas and the fraction of pigment stained islets, after injection of Monastral blue B at different times after either a single intravenous injection of streptozotocin (SZ) (160 mg/kg body-weight) or citric acid buffer (control)

Time after injection (h)	Treatment (*n*)		Serum glucose concentrations (mg/100 ml)		Total number of observed islets	Percent islets stained with Monastral blue B
			Before treatment[a]	After treatment[b]		
0.5	SZ	(5)	146± 2	166± 3*	257±13	0.5±0.1
0.5	Control	(5)	147± 8	179± 3	239±15	2.2±0.9
18	SZ	(5)	187± 3	49±10***	86±31	4.9±1.8
18	Control	(5)	171± 6	167±13	167±32	1.3±0.4
24	SZ	(6)	189± 4	133±28	105±12**	18.5±6.1*
24	Control	(7)	183± 7	169± 6	263±34	3.4±1.5
72	SZ	(5)	147±12	291±42***	35± 7***	76.2±9.0***
72	Control	(5)	156± 5	129± 7	268±32	1.5±1.5
192	SZ	(5)	175± 3	521±65***	19± 4***	86.2±8.5***
192	Control	(5)	172± 8	188±66	212±20	1.6±0.7

[a] Taken before injection

[b] Taken as indicated in the first column at different times after the injection of SZ or citric acid buffer. After the second blood sample Monastral blue B was injected, the animals were killed 60 min later and the pancreases treated for visualization of the islets. Values are given as means ± SEM for (*n*) animals and groups of SZ-treated animals were compared with the corresponding control group using Student's unpaired *t*-test. * $P<0.05$; ** $P<0.01$; *** $P<0.001$

Table 2. Pancreatic islet histology rank in (*n*) male C57BL/KsJ mice at different times after either a single intravenous injection of streptozotocin (SZ) (160 mg/kg body-weight) or citric acid buffer (control)

Islet morphology rank		A	B	C	D
SZ	(*n*)				
0.5 h	5	5	0	0	0
18 h	5	0	0	0	5
24 h	4	1	0	0	3
72 h	5	0	0	0	5
192 h	5	0	0	0	5
Control	(*n*)				
0.5 h	5	5	0	0	0
18 h	5	5	0	0	0
24 h	5	5	0	0	0
72 h	5	3	0	0	2
192 h	5	5	0	0	0

A: Normal islet structure; **B:** Some mononuclear cell in filtration in the peri-insular area; **C:** Heavy infiltration into a large number of islets i.e. insulitis; **D:** Diabetic appearance (only a few small islets remaining)

Table 3. Serum glucose concentrations of male C57BL/KsJ mice, total number of observed islets in approximately half the pancreas and the fraction of pigment stained islets, after injection of Monastral blue B at different times after either multiple intraperitoneal injections of streptozotocin (SZ) (40 mg/kg body-weight) or citric acid buffer (control)

Time after first injection (days)	Treatment; number of injections	(*n*)	Serum glucose concentrations (mg/100 ml)		Total number of observed islets	Percent islets stained with Monastral blue B
			Before treatment[a]	After treatment[b]		
2	SZ; 2	5	156± 4	178± 5**	223± 8	1.3± 0.4
2	Control; 2	5	160± 7	118±17	209±17	0.9± 0.3
3	SZ; 3	6	161±16	124± 4	184±19***	17.4± 3.4*
3	Control; 3	7	162± 8	139± 5	303±12	5.7± 1.9
7	SZ; 5	8	189± 5	242±10***	47± 7***	60.7±10.7**
7	Control; 5	10	171± 6	182± 6	189±12	2.0± 0.8
10	SZ; 5	7	161± 4[c]	214±17*[c]	59±10**	53.3±12.2**
10	Control; 5	9	155± 6[c]	166± 5[c]	152±17	6.4± 1.9
14	SZ; 5	10	165± 6	329±34***	37±10***	33.5± 8.6**
14	Control; 5	8	161± 8	181± 8	209±11	3.6± 1.4

[a] Taken before any injections were given

[b] This blood sample was taken as indicated in the first column at different times after the first injection of SZ or citric acid buffer. After the second blood sample Monastral blue B was injected, the animals killed 60 min later and the pancreases treated for visualization of the islets.

[c] The number of serum glucose determinations was 17, since a blood sample was taken on day 10 from the animals killed on day 14. Values are given as means ± SEM for (*n*) animals and groups of SZ-treated animals were compared with the corresponding control group using Student's unpaired *t*-test. * $P<0.05$; ** $P<0.01$ and *** $P<0.001$

Table 4. Pancreatic islet histology rank in (*n*) male C57BL/KsJ mice at different times after either two to five intraperitoneal injections of streptozotocin (SZ) (40 mg/kg body-weight) or citric acid buffer (control)

Islet morphology rank		**A**	**B**	**C**	**D**
SZ	(*n*)				
2 days	5	4	0	0	1
3 days	5	5	0	0	0
7 days	8	7	0	0	1
10 days	7	0	2	3	2
14 days	10	0	3	5	2
Control	(*n*)				
2 days	5	5	0	0	0
3 days	5	4	1	0	0
7 days	10	10	0	0	0
10 days	9	8	1	0	0
14 days	8	8	0	0	0

A: Normal islet structure; **B:** Some mononuclear cell infiltration in the peri-insular area; **C:** Heavy infiltration in a large number of islets i.e. insulitis; **D:** Diabetic appearance (only a few small islets remaining)

(Table 3). At this time there was a reduction in the number of islets observed and a markedly increased Monastral blue B staining of the islet vasculature. It is noteworthy that a decrease in the number of visible islets and an increased uptake of the blue colloidal pigment were observed already after three low-dose injections of SZ without any corresponding elevation of the serum glucose concentration. Light microscopic examination showed a mononuclear cell infiltration in the pancreatic islets of the multiple SZ-treated mice first on day 10 (Table 4). A number of the SZ-treated animals also showed a pronounced islet degeneration at different time intervals after the first injection (class D). The histological appearance of the pancreases of the citric acid buffer treated control animals was essentially normal.

Discussion

The results of the present study show that Monastral blue B administration in vivo in combination with the freeze-thawing technique to visualize the pancreatic islets (Jansson and Hellerström 1981) is a useful method for demonstrating a reduction in number of visible islets and increased vascular permeability caused by SZ administration. Indeed, increased islet vascular staining was observed before the animals had become diabetic by either of the two modes of SZ treatment. The blood vessels stained by the Monastral blue B pigment are those with increased endothelial permeability but with an intact basement membrane (Joris et al. 1982). In most tissues the postcapillary venules are the vessels which most intensively accumulate the pigment. It is to be noted in this context that the pigment was seen almost exclusively in the periphery of the islets indicating that the vessels in this area are of a venular type. This finding is in good agreement with previously published morphological studies demonstrating the presence of efferent vessels of a venular type in this area (Thiel 1954; Bonner-Weir and Orci 1982).

The present data show that both a single diabetogenic dose of SZ and multiple low doses induced increased permeability of the vascular system in the islets. In the case of a single high dose of SZ an acute B-cytotoxic effect was achieved as manifested clinically by a severe hypoglycaemia 18 h after the injection, later followed by manifest diabetes. This hypoglycaemic phase has been interpreted as due to a passive leakage of insulin from damaged B-cells (Rerup and Tarding 1969). It is conceivable that such cell destruction may stimulate the migration of histiocytic cells involved in the disposal of cell debris, a process which may be accompanied by the local secretion of substances mediating an increased vascular permeability. The present findings of a reduction in number of visible islets, increased islet vascular permeability, but no signs of insulitis on day three in the multiple low-dose injected animals suggest that the low doses of SZ exerted a significant toxic action on the B-cells as is the case after a single high dose of SZ, but at this time point the B-cell destruction was not sufficient to result in hyperglycaemia. It is therefore plausible that the same cellular reactions may have caused the increased islet vascular staining in both groups of SZ-treated mice. This supports the view that a direct B-cytotoxic

action of SZ is of importance for the development of hyperglycaemia in the multiple SZ model (Bonnevie-Nielsen et al. 1981; Sandler 1984). However, an immune component may well also contribute to a further loss of insulin-producing cells after the multiple SZ-doses which eventually will lead to hyperglycaemia. Moreover, it has been shown that immune reactions and a concurrent insulitis may be of pathogenetic significance for the development of hyperglycaemia in animals sensitized to SZ which were later reexposed to the drug (Sandler and Andersson 1981; Kim and Steinberg 1984). It should be noted that from the present study it cannot be deduced to what extent SZ-induced insulitis contributes to the development of hyperglycaemia in the multiple SZ-model. Nevertheless, it has been postulated that human Type I diabetes mellitus may develop after sequential environmental insults with inflammatory lesions caused by toxins, viruses or an activation of autoimmune processes (Toniolo et al. 1980).

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