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Alloxan-induced diabetes in the mouse: Time course of pancreatic B-cell destruction as reflected in an increased islet vascular permeability*

L. Jansson and S. Sandler

Department of Medical Cell Biology, Uppsala University, Biomedicum, P.O. Box 571, S-751 23 Uppsala, Sweden

Summary. The extent to which injections of the pancreatic B-cytotoxin alloxan in C57BL/Ks mice induced an increase in islet vascular permeability, and the time course of this increase, were studied. The vascular permeability was monitored by administration of the dye Monastral blue B, which is entrapped in leaky blood vessels with intact basement membranes. The islets were visualized by a freeze-thawing technique which allows identification of stained islets. Not until four hours after the alloxan injections was there an increase in islet uptake of Monastral blue B when compared with saline-treated control animals. Thereafter the islet staining increased further. The process was accompanied by gradual development of hyperglycaemia and a reduction of number of the islets identified in the pancreatic preparations. It is concluded that alloxan causes an increase in islet vascular permeability, which appears to become manifest at a later stage than the cytotoxic B-cell degeneration.

Key words: Alloxan – Diabetes – Pancreatic islets

Introduction

In a recent study it was demonstrated that the diabetogenic drug streptozotocin (SZ) caused an increase in vascular permeability in mouse pancreatic islets both when given as a single high dose and as multiple low doses (Sandler and Jansson 1985). The islet vascular permeability was monitored by injections of Monastral blue B, a pigment which becomes entrapped in leaky blood vessels with intact basement membranes (Joris et al. 1982). The islets were visualized without staining of the non-fixed pancreas by means of a freeze-thawing procedure (Jansson and Hellerström 1981). The results confirmed that SZ exerted a direct cytotoxic action on the pancreatic B-cells and suggested that this process may lead to the release of substances mediating an increase in vascular permeability.

Alloxan is a B-cytotoxin most commonly used for induction of experimental diabetes. It has been suggested that both it and SZ have a similar mode of action (Yamamoto et al. 1981) and that knowledge of this mechanism(s) may shed light on the pathogenesis of human Type I diabetes mellitus. In the present investigation we have studied the islet vascular permeability at different points of time following administration of a single diabetogenic dose of alloxan in mice. It has been demonstrated that during spontaneous conversion in vitro, alloxan generates free oxygen radicals (Lagercrantz and Yhland 1963; Grankvist 1981; Asayama et al. 1984). Furthermore, the selective vulnerability of the islet B-cells compared with other cell types has been attributed to a high rate of uptake of alloxan in the B-cells (Malaisse et al. 1982). Thus, it could be speculated that noxious free radicals might damage blood vessels in the islets in addition to destroying the B-cells (Cohen and Heikkila 1974; Grankvist et al. 1979; Fischer and Hamburger 1980).

Materials and methods

Animals. Male inbred C57BL/Ks mice, aged 12–16 weeks, originally obtained from a colony at the Jackson Laboratories, Bar Harbor, ME USA, were used. The animals were allowed free access to tap water and pelleted food (Ewos-Anticimex,

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Offprint requests to: S. Sandler at the above address

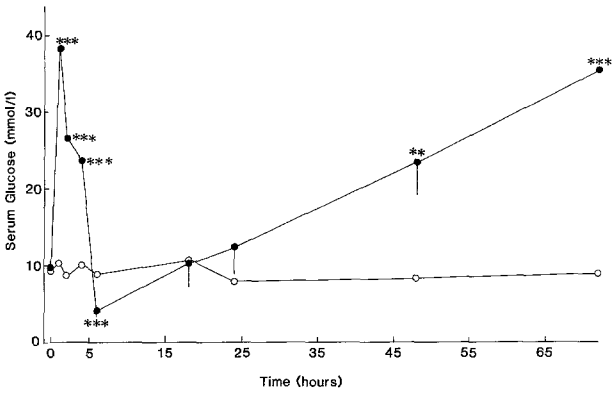

Fig. 1. Serum glucose concentrations in male C57BL/Ks mice at different points in time following an intravenous injection of alloxan (75 mg/kg body weight; *closed circles*) or saline (9 g/l w/v; *open circles*). Data are given as means and SEM is indicated when exceeding 10% of the mean. A total of 47 animals received alloxan and 42 animals were correspondingly injected with saline and their serum glucose concentrations before the injection is given at time 0. At the following times the serum glucose concentrations of 4–8 animals were measured prior to the Monastral blue B injections upon killing. ** and *** denote $P<0.01$ and $P<0.001$ respectively, using the unpaired Student's t test

Type R3; Ewos, Södertälje, Sweden) throughout the experimental period. Before any treatment was given, a blood sample was taken by retroorbital sinus puncture. Serum glucose concentrations in these samples were determined by a glucose oxidase method (Glucose Analyzer 2, Beckman Instruments, Fullerton, CA, USA).

After the first blood sample had been drawn, the experimental animals were injected with a diabetogenic dose of alloxan (Sigma Chemicals, St Louis, MO, USA) dissolved in saline (75 mg/kg body weight). Animals receiving an equal volume of saline (200 µl) served as controls. A second blood sample was taken at different time intervals (1, 2, 4, 6, 18, 24, 48 or 72 h) after the injection of alloxan or saline.

Determination of islet vascular permeability. After the second blood sample had been obtained the mice were injected intravenously with a suspension of 0.1 ml of Monastral blue B composed of 3% (w/v) phthalocyanine blue pigment dissolved in saline (0.85% w/v) (Sigma Chemicals) and they were killed 60 min later by cervical dislocation. The pancreatic glands were quickly removed and dissected free from non-pancreatic tissue. The glands were weighed and divided into approximate halves, which were weighed again before further processing. For visualization of the pancreatic islets the freeze-thawing method described by Jansson and Hellerström was used (1981). For this purpose one half of each pancreas was cut further into small pieces, each weighing approximately 10–15 mg, and squeezed between two object slides. The preparations were frozen and stored for at least 2 h 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 which was also equipped with bright field illumination (Wild Heerbrugg Ltd., Heerbrugg, Switzerland), at 400× magnification. The Monastral blue B pigment entrapped within the vessel walls could be visualized better, however, in bright field illumination. The practical procedure was therefore to first 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 weighed preparation and the fraction of islets containing blue pigment was calculated, as was the number of observed islets per pancreatic wet weight. Only islets with diameters exceeding 50 µm were counted, since islets of smaller size were difficult to distinguish from connective tissue septa.

Light microscopy. The remaining half of each pancreas was fixed in Bouin's solution and embedded in paraffin. Sections (7 µm thick) were cut and stained with haematoxylin and eosin. The pancreatic sections were coded so that the examiner was unaware of the origin of the section. The pancreatic islets were examined histologically for degenerative changes in the islet cells and for round cell infiltration around and within the islets. The islet histology was arbitrarily ranked into to five classes (A–E): Class A denotes normal islet morphology; class B a low degree of mononuclear cell infiltration, especially in the periinsular area; class C heavy infiltration with mononuclear

Table 1. Number of observed islets per pancreatic wet weight and the fraction of pigment-stained islets in the pancreatic glands of male C57BL/Ks mice, following injection of Monastral blue B at different times after a single intravenous injection of alloxan (75 mg/kg body weight) or of saline (9 g/l; 200 µl)

Time after injection (h)	Number of observed islets (islets/mg pancreas)		Percent islets stained with Monastral blue B (%)	
	Alloxan	Saline	Alloxan	Saline
1	2.3 ±0.22 (7)	1.9±0.15 (7)	2.7±0.61 (7)	3.1±1.7 (7)
2	1.9 ±0.31 (8)	2.2±0.17 (8)	1.9±0.31 (8)	2.5±0.74 (8)
4	1.9 ±0.13 (6)	2.0±0.07 (7)	8.1±1.5 (6)**	2.6±0.64 (7)
6	2.1 ±0.17 (7)	2.1±0.13 (7)	29 ±2.2 (7)***	5.5±2.7 (7)
18	1.0 ±0.02 (4)**	2.0±0.23 (4)	40 ±2.3 (4)***	1.2±0.29 (5)
24	0.68±0.07 (8)**	2.0±0.37 (6)	30 ±4.2 (8)***	2.0±0.56 (6)
48	0.28±0.14 (6)***	2.0±0.12 (6)	33 ±7.7 (6)**	4.3±1.9 (6)

At different times following either alloxan or saline administration Monastral blue B was injected intravenously; the animals were killed 60 min later and the pancreas was weighed and treated for visualization of the islets as described in Material and Methods. Values are given as means±SEM for (n) animals, and groups of alloxan-treated animals are compared with the corresponding control group using Student's unpaired t test. ** $P<0.01$ and *** $P<0.001$

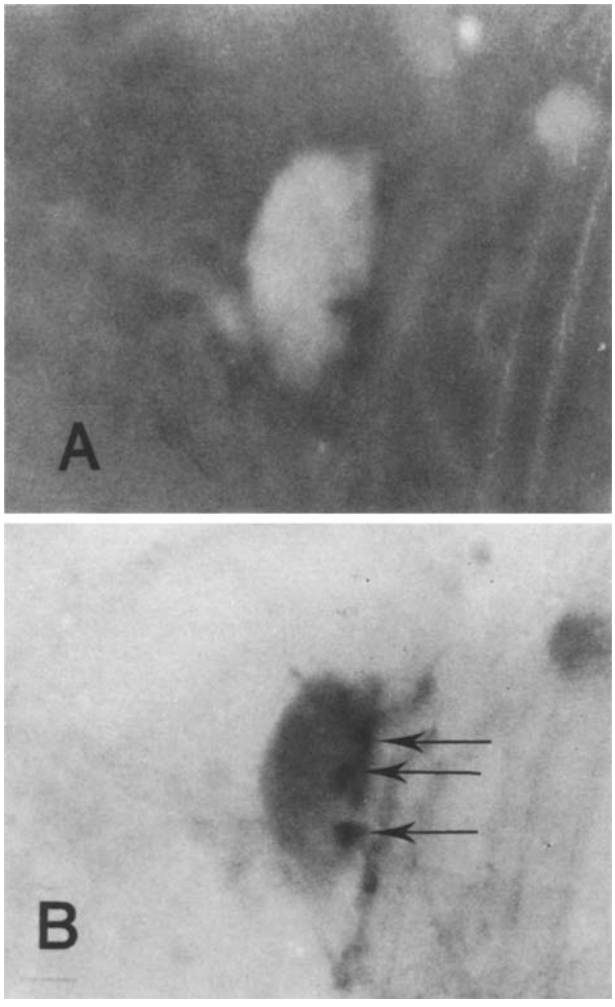

Fig. 2. Stereo micrograph of a pancreatic islet in dark field illumination **A** in a frozen-thawed preparation of a pancreas six hours after injection of alloxan. Staining of the islet with Monastral blue B pigment is confirmed in bright field illumination (**B**; *arrows* point to dark spots). Magnification 530 ×

cells in a majority of islets; class D only a few residual islets displaying cellular disarray and pyknotic nuclei; and class E islets with a normal shape but dilated capillaries, widened intercellular spaces and some nuclear pyknosis (Fig. 3). The morphological classifications A–D have recently been illustrated elsewhere (Sandler and Andersson 1985).

Statistical analysis. All values are expressed as means ± SEM. For comparing groups of data, the unpaired Student's two-tailed *t* test was used.

Results

Development of hyperglycaemia

Alloxan rapidly induced a transient pronounced peak of hyperglycaemia (Fig. 1). However, after six hours the alloxan-treated mice exhibited hypoglycaemic serum glucose concentrations when compared with the saline-treated controls. Eighteen and 24 h after the injections there were no differences in the serum glucose concentrations between the two groups of animals, but thereafter the alloxan-treated mice became grossly hyperglycaemic. It is notable that between the 6th and 72nd h the serum glucose concentrations showed a linear elevation with time ($r = 0.999$; $P < 0.001$).

Table 2. Pancreatic islet histology rank in (*n*) male C57BL/Ks mice at different times after an intravenous injection of alloxan (75 mg/kg body-weight) or saline (9 g/l; 200 µl)

	(*n*)	Islet morphology rank				
		A	B	C	D	E
Alloxan						
1 h	7	1	0	0	1	5
2 h	8	0	0	0	0	8
4 h	6	0	0	0	4	2
6 h	7	0	0	0	6	1
18 h	4	0	0	0	4	0
24 h	8	0	0	0	8	0
48 h	6	0	0	0	4	2
72 h	5	0	0	0	3	2
Saline						
1 h	7	7	0	0	0	0
2 h	8	8	0	0	0	0
4 h	7	7	0	0	0	0
6 h	7	6	0	1	0	0
18 h	5	5	0	0	0	0
24 h	6	6	0	0	0	0
48 h	6	5	0	0	0	1
72 h	5	5	0	0	0	0

A Normal islet structure; *B* Some mononuclear cell infiltration in the periinsular area; *C* Mononuclear cell infiltration in a majority of islets, i.e. insulitis; *D* Only a few residual islets displaying cellular disarray and pyknotic nuclei; *E* Intact islet shape but dilatation of the islet blood vessels and intercellular spaces and some nuclear pyknosis (Fig. 2)

Islet vascular permeability

Using the freeze-thawing technique for visualization of the pancreatic islets it became evident that in the control group the number of visible islets per mg pancreatic wet weight was remarkably constant, i.e. at about 2.0 islets/mg pancreas (Table 1). In the pancreatic glands from the alloxan-injected animals, also, the number of visible islets remained similar during the first six hours after alloxan administration. The number of observed islets then gradually decreased to about 15% of that in the controls. After 72 h those islets possibly remaining were so diffuse that they could not be distinguished

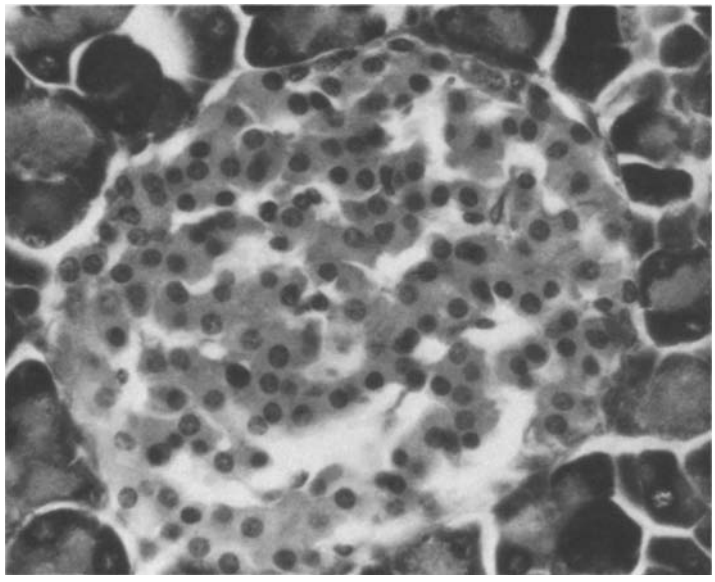

Fig. 3. Light micrograph of a pancreatic islet two hours after alloxan injection, showing widened intercellular spaces and dilatation of islet blood vessels. The morphology was ranked as class E. Haematoxylin and eosin, magnification × 500

from the rest of the pancreas in the frozen-thawed preparations. There were no differences in the total pancreatic wet weight between the experimental and control animals, at any time, the values lying in the range of 250–300 mg (data not shown).

In the control group the percentage number of islets stained with the Monastral blue B pigment was small and fairly constant at all time points (Table 1). The alloxan-treated animals showed a significant increase in the islet vascular staining (Fig. 2) beginning 6 h after the alloxan injection. This change thus occurred prior to any notable decrease in the number of visible islets. During the subsequent observation period (18–48 h) the proportion of visible islets stained was in the order of 30–40% in the alloxan-exposed animals.

Islet morphology

In all except two mice the islet morphology of the control animals was ranked as normal (Table 2). On the other hand, the islets of nearly all the experimental animals showed morphological alterations. Only one hour after the alloxan injection, pronounced structural changes in the islets were observed. Although the light microscopic appearance of the islet cells was intact, characteristic dilatations of intercellular spaces and islet blood vessels were evident as early as one hour after alloxan injection (Class E; Fig. 3). This appearance was gradually followed by nuclear pyknosis, and cellular necrosis with only a few small disintegrated islets visible in the sections (class D).

Discussion

The present findings show that administration of Monastral blue B is a sensitive method for demonstrating an increase in vascular permeability following early detection of an ongoing destructive process in the pancreatic islets. Indeed, leakage of the dye occurred following an alloxan injection before a reduction in the number of visible islets, as observed by the freeze-thawing technique, became evident. In accordance with this finding we also noted an increase in vascular permeability in mouse islets as early as day three after commencement of multiple low dose injections of SZ, i.e. when the serum glucose concentrations were normal (Sandler and Jansson 1985). Furthermore, the freeze-thawing method for visualization of islets more than 50 μm in size seems to give very reproducible results, as demonstrated by the calculations of the number of islets per mg pancreatic wet weight in the control group (Table 1).

The observation of a triphasic serum glucose response to the alloxan injections (Fig. 1) is in good agreement with previous reports (Rerup and Tarding 1969). The initial hyperglycaemic peak after administration of alloxan probably reflects accelerated glycogenolysis in the liver (Rerup and Tarding 1969), possibly caused by a rapid inhibition of insulin secretion and an enhanced catecholamine output. The subsequent hypoglycaemia is presumably attributable to a passive leakage of insulin from damaged B-cells. The gradual elevation of the serum glucose concentration after the hypoglycaemic phase at six hours was accompanied

by, and correlated with ($r=0.88$; $P<0.05$), a decrease in the number of visible islets in the alloxan-treated animals. In our previous study in which SZ was administered to C57BL/Ks mice, a hypoglycaemic phase also occurred but not until after 18 h (Sandler and Jansson 1985). At this time following an alloxan injection in the present investigation, the animals were apparently normoglycaemic. These differences in the dynamic response to alloxan and SZ in vivo suggest that the drugs act by different B-cytotoxic mechanisms. In line with this idea, we have demonstrated several differences in vitro in the function of isolated mouse islets that have been exposed to alloxan or SZ (Sandler and Swenne 1983; Sandler et al. 1984).

Although alloxan and SZ may act by different molecular mechanisms, both drugs induced an increase in vascular permeability in the pancreatic islets. However, it is conceivable that this following administration of both alloxan and SZ is mediated by a secondary local release of vasoactive substances in the islets during the destruction of B-cells. According to this hypothesis the increased vascular permeability would not be a primary effect of either alloxan or SZ. If alloxan had generated free radicals which damaged the islet blood vessels primarily, increased vascular permeability should already have been evident one hour after the alloxan injections. In accordance with the present results, Grankvist and Rooth (1985) recently reported that up to 60 min after an intravenous injection of alloxan in mice, there was no increase in the islet capillary permeability as determined by fluorescence microscopy in vivo.

From examinations of the islet morphology after the administration of alloxan, it was clear that this drug rapidly induced structural changes which in some respect were different from those caused by SZ (Sandler and Jansson 1985). Multiple low dose injections of SZ resulted in pancreatic insulitis (Class C), suggesting that an immune reaction directed against the islet cells had been provoked by SZ, but in the present study with single-dose alloxan no insulitis was observed. Alloxan, on the other hand, caused alterations within the islets, for example widened intercellular spaces and dilatations of islet blood vessels. It should be noted that these changes did not correlate with the increased islet vascular permeability as demonstrated with Monastral blue B staining. In accord with these findings, Lazaruz and Volk (1962) observed slight shrinkage of the B-cells with widening of pericapillary spaces in rabbit islets as the earliest changes after alloxan injection.

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