Pentamidine, A New Diabetogenic Drug in Laboratory Rodents

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Summary. The antiprotozoal drug, pentamidine, has been reported to induce hypoglycaemia associated with inappropriately high plasma insulin concentrations, followed by insulin-dependent diabetes mellitus. It has been suggested that this drug can be toxic to the islet B cell, inducing early cytolytic release of insulin leading to B cell destruction. In order to test this hypothesis, mouse and rat islets were incubated with pentamidine at concentration range of 5×10^{-11} to 5×10^{-3} mol/l and exposure times of 3–48 h. The B cell responses to glucose + theophylline and to arginine were suppressed by pentamidine, while insulin release in non-stimulatory conditions was

increased. These effects were dose-dependent, time-dependent and irreversible. They were significant for 5×10^{-7} mol/l pentamidine, which is a concentration relevant to therapeutic uses. These effects developed more slowly than the toxic effects of streptozotocin and alloxan at the same molar concentration in vitro. ⁵¹Chromium release and Trypan blue exclusion tests support the hypothesis that pentamidine produces islet cell necrosis.

Key words: Pentamidine, diabetes mellitus, insulin secretion, B cell, diabetogenic drugs.

The antiprotozoal drug, pentamidine, is an aromatic diamidine used for the chemotherapy of trypanosomiasis, leishmaniasis and *Pneumocystis Carinii* pneumonitis [1, 2]. Hypoglycaemic attacks have occurred in 91 cases [3–5]. Inappropriately high plasma insulin concentrations have been documented in some of these patients [3]. Diabetes mellitus has also been observed in 31 reported cases [6]. In eight of these subjects, diabetes developed after the occurrence of hypoglycaemia and was usually insulin-dependent. For these reasons we have suggested that pentamidine is toxic to B cells, inducing early cytolytic release of insulin, B cell destruction, insulinopenia and diabetes [3]. In order to check this hypothesis, mouse and rat islets were exposed to pentamidine in vitro, and basal and stimulated insulin release was measured.

Material and Methods

Mouse and Rat Islet Preparations

Adult DBA/2 mice and Wistar rats (CNRS, Orléans, France) fed ad libitum were used. Islets were prepared as described by Lacy and Kostianovsky [7], pancreatic tissue from five mice or one rat pancreas being treated with 15 mg collagenase (150 mU/mg; Boehringer, Mannheim, FRG). After manual shaking, islets were picked up under

a dissecting microscope (Nikon, SMZ 2, Tokyo, Japan) and transferred to Petri dishes set on ice. Islets were then either put on incubation plates or perfused directly.

Incubation Experiments

Five islets were suspended in each well of Falcon microtest plates (Becton-Dickinson, Rockville, Maryland, USA) in MEM Eagle medium (Flow Laboratories, Rockville, Maryland) supplemented with 10% fetal calf serum, glutamine (2 mmol/l), sodium pyruvate (1 mmol/l), non-essential amino-acids (0.814 mg/l), streptomycin (100 µg/ml), penicillin (100 mU/l). This medium, containing glucose (5.5 mmol/l), was equilibrated with O₂ and CO₂ (95%–5%) and the final pH was 7.4. The medium was called basal medium.

Pentamidine dimethane sulphonate (Specia, Paris, France) was dissolved in basal medium. Freshly isolated islets were incubated in basal medium (5 islets/250 µl) for 24 h at 37 °C with O₂ and CO₂ (95% and 5%), in the presence of pentamidine 5×10^{-11} to 5×10^{-3} mol/l. Islets were also exposed to pentamidine 5×10^{-3} and 5×10^{-5} mol/l for 3–48 h.

At the end of the incubation period, islets were washed twice and then exposed to the basal or stimulatory media (250 µl/well) for 30 min. The standard stimulatory medium consisted of the basal medium, except for the addition of glucose (16.5 mmol/l) + theophylline (5 mmol/l). In other experiments, the basal medium was supplemented with arginine (20 mmol/l). Similar experiments were performed with sodium methane sulphonate (5×10^{-5} mol/l) in place of pentamidine dimethane sulphonate. To test the reversibility of pentamidine effects, islets were also exposed to pentamidine 5×10^{-5} mol/l for 24 h, washed three times and re-incubated for 24 h in a pentamidine-free medium, before the stimulation described above.

Fig. 1. Dose-dependency of pentamidine influence on B cells. Islets were incubated for 24 h in basal medium (glucose 5.5 mmol/l) with various concentrations of pentamidine, then washed and exposed for 30 min to either a basal or a stimulatory medium (glucose, 16.5 mmol/l + theophylline, 5 mmol/l). □ = insulin basal release and ▨ = stimulated release. Results are presented as mean $\pm$ SEM of five experiments. Asterisks at top of columns denote differences in stimulated IRI release between pentamidine-treated and control islets (**: $p < 0.001$; *: $p < 0.05$)

Direct Perfusion Experiments

Freshly isolated islets were placed in a flow-through perfusion system [8, 9]. Three perfusion media were prepared. The basal medium, derived from Krebs-Ringer physiological solution, contained the following components: NaCl (120 mmol/l), KCl (4.7 mmol/l), $MgSO_4$ (0.8 mmol/l), $CaCl_2$ (2.5 mmol/l), KH_2PO_4 (1.2 mmol/l), $NaHCO_3$ (25 mmol/l), human serum albumin (0.60%; Centre National de Transfusion Sanguine, Paris, France), aprotinin (2,000 μ /ml; Choay, Paris, France), glucose (5 mmol/l), sodium pyruvate, fumarate and glutamate (5 mmol/l each; Sigma, St. Louis, Missouri, USA). The final pH was adjusted to 7.4 by adding 4–6 drops of normal sodium hydroxide. The stimulatory medium was essentially similar, except for glucose concentration which was 20 mmol/l and the addition of theophylline (5 mmol/l). The pentamidine-containing medium was similar to the basal medium, except for the addition of pentamidine (10^{-4} mol/l). Final pH was adjusted to 7.4 after complete dissolution of the drug. All three media were warmed to 37°C and gassed with a mixture of $O_2 + CO_2$ (95% + 5%) before and during perfusion experiments, the pH and temperature being repeatedly monitored. Chambers were perfused at a constant flow rate of 1 ml/min. Experiments lasted for a maximum of 160 min. Samples were collected at predetermined intervals of time, cooled on ice and frozen at -20°C until assayed. Viability of islets was tested at the end of all experiments by the Trypan blue exclusion test.

Incubation + Perfusion Experiments

Freshly isolated islets were incubated for 18 h in supplemented MEM Eagle medium in the presence or in the absence of pentamidine (10^{-4} mol/l), then transferred to the perfusion chambers, perfused in basal medium for 40 min and stimulated by glucose (20 mmol/l) + theophylline.

Trypan Blue Exclusion Test

Islets were exposed to a 0.5% Trypan blue isotonic solution for 1 min, then examined under a dissecting microscope (Nikon SMZ 2, Tokyo, Japan) under a white episcopic light, using $\times 40$ magnification.

^{51}Cr Chromium Cytotoxicity Assay

Dispersed islet cells were prepared as described previously [10] and their viability was tested by the Trypan blue exclusion test; only suspensions with at least 90% viable cells were retained. Cells were incubated at 37°C for 1–5 h with 80 mCi of $Na_2^{51}Cr O_4$ (CNTS, Paris, France), washed twice and then incubated for 24 h in the presence of pentamidine at a concentration range of $5 \cdot 10^{-11}$ to $5 \cdot 10^{-3}$ mol/l. The supernatants were counted in a gamma scintillation spectrometer (Packard, Chicago, Illinois). The ^{51}Cr release was compared with that of cells lysed with distilled water.

Assessment of Hormone Damage

During incubations, the insulin may have been partly damaged. This damage might have been reduced either by pentamidine, which is chemically close to the antiprotease molecule benzamidine [11], or augmented by the lysosomal enzymes of necrotic islet cells.

To assess the extent of insulin damage, ^{125}I -insulin (500 μ Ci/ μ g – CNTS, Paris, France) was incubated with islets and pentamidine for 2 and 48 h. Supernatants were then treated by dextran-coated charcoal [12]. It was assumed that the damaged hormone (i.e. free iodine and small peptides) corresponded to the fraction which was not retained by the dextran-coated charcoal.

Determination and Expression of Results

Insulin (IRI) was determined by radioimmunoassay [13] using charcoal for the separation of free and bound hormone, purified rat insulin as a standard and porcine ^{125}I -insulin as a tracer. It was verified that pentamidine (10^{-3} mol/l) did not interfere with the assay. Results are expressed as mean $\pm$ SEM. Statistical significance of differences was analysed by using the Student's t-test for non-paired values.

Results

Influence of Pentamidine on the B Cell Responses to Stimuli

Incubation Experiments: Dose-Dependency: As shown in Figure 1, mouse islets, incubated for 24 h in the basal control medium, responded to the stimulation by glucose (20 mmol/l) + theophylline (5 mmol/l) by a significant IRI release (0.53 ± 0.03 versus 0.03 ± 0.01 mU/30 min per five islets in basal medium, $n = 6$; $p < 0.001$). Pentamidine (5×10^{-9} and 5×10^{-11} mol/l) did not modify significantly this B cell response. But this response was significantly lower than control in islets treated by pentamidine 5×10^{-7} mol/l. This response was abolished for concentrations of 5×10^{-5} and 5×10^{-3} mol/l.

Time-Dependency: this was analysed for pentamidine concentrations of 5×10^{-5} and 5×10^{-3} mol/l (Fig. 2). While a significant B cell response to stimulus was observed in all control experiments (Fig. 2, upper panel), no response occurred in the case of islets exposed to pentamidine 5×10^{-5} mol/l and 5×10^{-3} mol/l, from the shortest (i.e. 3 h) to the longest (48 h) time of exposure (Fig. 2, central and lower panels). The B cell response from the pentamidine-treated islets was in all cases significantly lower than that from control islets in the same stimulatory medium. Thus suppression of IRI release was proportionate to the duration of exposure to the drug.

Fig. 2. Time-dependency of the pentamidine influence on B cells. Islets were incubated for 3–48 h in basal medium (upper panel) or in basal medium containing pentamidine (5×10^{-5} mol/l) (central panel) or (5×10^{-3} mol/l) (lower panel). The islets were then washed and exposed to a basal or stimulatory medium as depicted in legend of Figure 1. Results are presented as in Figure 1. Experiments were performed in sextuplicate

Fig. 3. Irreversibility of the pentamidine influence on B cells. Islets were incubated for 24 h in basal medium or in basal medium containing pentamidine 5×10^{-5} , then washed and incubated for a further 24 h in a pentamidine-free medium. They were then exposed for 30 min to either a basal or a stimulatory medium, as depicted in legend of Figure 1. Results are presented as in Figure 1. Experiments were performed in sextuplicate

When islets had been pre-incubated for 24 h with pentamidine (5×10^{-5} mol/l), and then washed and allowed to stay for a further 24 h in a pentamidine-free medium, there was still no B cell response to glucose + theophylline (Fig. 3).

When islets were treated with sodium methane sulphonate (5×10^{-5} mol/l) for 24 h instead of pentamidine dimethane sulphonate, basal and stimulated IRI release was not different from control values: 0.03 ± 0.01 and 0.43 ± 0.08 mU/30 min per five islets, respectively, versus 0.03 ± 0.01 and 0.43 ± 0.10 mU/30 min per five islets respectively for the untreated control islets. When pentamidine-treated islets (5×10^{-3} mol/l for 24 h) were stimulated by arginine (20 mmol for 30 min), no B cell response occurred: the basal value being 0.10 ± 0.02 mU/30 min per five islets and the stimulated value 0.09 ± 0.02 mU/30 min per five islets (versus 0.04 ± 0.01 and 0.15 ± 0.05 respectively in control experiments).

Incubation + perfusion: Islets were incubated for 18 h in supplemented MEM Eagle medium as described above in the presence or the absence of pentamidine (10^{-4} mol/l), then transferred to the perfusion system and stimulated by arginine (20 mmol/l) + theophylline (5 mmol/l). The B cell response in pentamidine-treated rat islets was dramatically lower than in control islets (Fig. 4, upper panel). In contrast, somatostatin release was similar to that in control islets (Fig. 4, lower panel). Similar results were obtained as regards the B cell response to glucose (20 mmol/l) + theophylline (5 mmol/l) from mouse or rat islets similarly treated by pentamidine then perfused in vitro (data not shown).

Direct perfusion experiments: Freshly isolated rat islets were perfused immediately, exposed to pentamidine (10^{-4} mol/l) for 40 min, then stimulated by glucose (20 mmol/l) + theophylline (5 mmol/l). The mean IRI release following pentamidine exposure was 73 ± 11 versus 55 ± 8 μ U/min per 40 islets in control perfusions ($n=10$; NS), but for the stimulatory period the mean increase in IRI secretion was lower in the pentamidine-treated islets than in the control islets (0.04 ± 0.01 versus 0.08 ± 0.01 mU/min per 40 islets; $p < 0.05$). The switch from basal to pentamidine medium induced no massive IRI release during a 120 min observation period.

Influence of Pentamidine on Non-stimulated IRI Release

Incubation experiments: As already noted in Figures 1 and 2, pentamidine exerted complex influences on non-stimulated IRI release. In a dose-response study, pentamidine (5×10^{-9} and 5×10^{-11} mol/l) did not alter the basal IRI release in mouse islets at the end of the 24 h-incubation period (Table 1). At concentrations of 5×10^{-7} to 5×10^{-3} mol/l, IRI release was dramatically increased.

Table 1. Dose-response to pentamidine in basal medium (glucose 5.5 mmol/l)

	Control islets	Pentamidine treated islets (mol/l)				
		5×10^{-11}	5×10^{-9}	5×10^{-7}	5×10^{-5}	5×10^{-3}
Insulin release (mU/24 h per 5 islets)	0.10 ± 0.02	0.10 ± 0.01	0.12 ± 0.01	$1.35^a \pm 0.09$	$1.22^a \pm 0.03$	$0.45^a \pm 0.03$

Results expressed as mean $\pm$ SEM of six experiments in each group.

Mouse-islets were incubated in basal medium for 24 h in the presence of pentamidine at various concentrations after which insulin was assayed in supernatant in absence of any further stimulation. ^a $p < 0.001$ statistically significant difference from control islets

Fig. 4. Kinetics of the B and D cell responses to arginine+theophylline (incubation-perfusion experiments). Rat islets were pre-incubated with pentamidine (10^{-4} mol/l) for 18 h, then placed in perfusion chambers and perfused with basal medium (glucose, 5 mmol/l)+theophylline (5 mmol/l). $\circ$ — $\circ$ =Control experiments (six experiments) and $\bullet$ — $\bullet$ =pentamidine-experiments (six experiments). The hatched bar symbolizes the arginine+theophylline stimulation. Results are presented as mean $\pm$ SEM

Islets were also serially incubated for 3–48 h to analyse the influence of the duration of the incubation period (Table 2). The IRI concentration in basal medium did not vary significantly. In the presence of the drug, the IRI level was markedly increased for the three shortest exposure times.

Modifications in hormone damage might have contributed to these complex effects of pentamidine.

125 I-labelled insulin was incubated with basal medium plus islets in the absence and in the presence of pentamidine (5×10^{-3} mol/l) and aprotinin (20,000 U/ml). Incubations were conducted at 37 °C for 2–48 h. Aliquots of supernatants were then added to 500 μ l dex-

Table 2. Time dependency of insulin release after exposure to pentamidine (10^{-3} mol/l)

Insulin release (mU/5 islets per time of incubation)	Incubation time (h)			
	3	18	24	48
Control islets	0.09 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.08 ± 0.01
Pentamidine-treated islet	1.90 ± 0.05^a	1.55 ± 0.04^a	0.45 ± 0.03^a	0.08 ± 0.01

Results expressed as mean $\pm$ SEM of six experiments in each group

Islets were maintained in a non-stimulatory medium (glucose 5.5 mmol/l) in the presence of pentamidine. Then insulin was assayed in supernatant in absence of any further stimulation. ^a $p < 0.001$ statistically significant difference between pentamidine-treated islets and the corresponding control group

tran-coated charcoal in the presence of 50 μ l normal sheep serum. The percentage radioactivity not retained by dextran-coated charcoal was increased in the supernatants after 48 h in the presence of pentamidine-treated islets: 57% versus 13% in case of control islets or tracer alone. This damage was slightly reduced (52% instead of 57%) by the addition of aprotinin. This suggests that the increase in basal IRI concentration observed after pentamidine exposure was not due to a reduction of hormone damage. On the contrary, the damage of tracer was increased, presumably due to cell necrosis and release of intracellular proteases. Similar results were obtained when 125 I-glucagon was incubated with islets and pentamidine.

Characterization of B Cell Necrosis

51 Chromium release test: The chromium release from dispersed islet cells incubated for 24 h in basal medium with pentamidine (5×10^{-7} to 5×10^{-3} mol/l) was significantly higher than control values (Fig. 5). This increase was dose-dependent.

Trypan blue exclusion test: Dark blue islets were, in all instances, easily distinguishable from the bright white islets. The percentage of blue islets was $< 1\%$ in the case of control islets either freshly isolated or incubated for 18 h in MEM Eagle medium. It was also $< 1\%$ when islets were exposed to pentamidine (10^{-4} mol/l) for 120 min or less. This percentage increased progressively

Fig. 5. Chromium release from pentamidine-treated dispersed islet cells. Freshly prepared islet cells were treated with ⁵¹Chromium, then re-incubated for 24 h with pentamidine at various concentrations. The radioactivity in the supernatants was counted and compared with the ⁵¹Chromium release from control cells. These had been similarly prepared, incubated for 24 h in the absence of pentamidine, and then lysed by distilled water. Experiments were performed in triplicate. ***p* < 0.001

and reached 55% of islets after 5 h. It was 95–100% after a 18-h long exposure to the drug at a concentration of 10⁻⁴ mol/l. This time-course differed greatly from those obtained after exposure to streptozotocin or alloxan, the toxic effects of which were much faster than those of pentamidine at the same concentrations.

Discussion

Our data suggest that pentamidine exerts two distinct influences on the B cells: (1) an impairment of B cell response to glucose and arginine detectable as early as 1 h after exposure to the drug, and (2) a cytolytic process strongly suggested by ⁵¹Chromium release, the Trypan blue test and excessive release of insulin in non-stimulatory conditions. This leakage may be similar to that observed when alloxan and streptozotocin are used [14, 15], albeit less acute in the case of pentamidine than with alloxan [15]. Streptozotocin, added to dispersed islet cells incubated in vitro, induces a pattern very similar to that obtained with pentamidine, i.e. IRI release in non-stimulatory conditions and no response to glucose + theophylline (data not shown).

The clinical relevance of our experiments is substantiated by: (1) the time-course of published clinical events, where early hypoglycaemic attacks precede the onset of diabetes mellitus [3] and (2) the concentrations of pentamidine measured in plasma and tissues of treat-

ed patients (i.e. 10⁻⁶ mol/l) which correspond to those toxic for islets in vitro [16, 18]. Furthermore, in several patients with pentamidine-induced diabetes, renal insufficiency was present [3]. As pentamidine is partly excreted by the kidney, this condition may favour the accumulation of the drug in the tissues. In some cases, successive treatment courses have an additive effect on the impairment of glucose tolerance [3, 20]. Although slight variations in glucose tolerance have been described in pentamidine-treated rabbits [21], no animal model of pentamidine-induced diabetes mellitus is currently available in vivo in laboratory rodents.

Two pentamidine salts are currently used in therapeutics: isethionate and mesylate (or dimethane sulphonate). The latter may be the more diabetogenic [6, 19, 20]. Since isethionate can alter hormone release by itself [22], it is important to note that: (1) pentamidine dimethane sulphonate was used in the present study, and (2) sodium methane sulphonate had no effect. Further work is needed to clarify the mechanism of action of the drug on islet B cells at the molecular level. It has been shown in protozoa and bacteria that pentamidine interferes mainly with protein synthesis and ribosomal activities [23–26], with mitochondrial structure and oxidative metabolism [26–29]. It also impairs several enzymatic systems, including that of kallikrein [30], which may be involved in the insulin secretory process [31].

Islet B cells were specifically affected by pentamidine in the concentration range studied here, while D cell function was not impaired. Further work, currently in progress, suggests that rat hepatocytes and some non-pancreatic cultured cell lines are less sensitive to the pentamidine toxicity than islet B cells. If confirmed, this would provide a new tool for the analysis of B cell metabolism and further potential for the medical treatment of insulinomas.

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