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Selective effects of pentamidine on cytosolic and granule-associated enzyme release from zymosan-activated human neutrophilic granulocytes

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Abstract—Therapeutic concentrations (0·3–1·5 mg L^{-1}) of pentamidine isethionate, normally obtainable in-vivo after parenteral administration of the drug, did not affect the in-vitro activity of the enzymes lysozyme, β-glucuronidase or myeloperoxidase released from zymosan-activated human neutrophilic granulocytes. At concentrations of 0·7, 1·1 and 1·5 mg L^{-1}, activity of cytosolic enzymes lactate dehydrogenase and glucose-6-phosphate dehydrogenase were reduced relative to untreated cells ($P < 0.001$ and $P < 0.01$, respectively), but not in a dose-dependent fashion. Cell viability, as determined by dye-exclusion remained unaffected.

Pentamidine (1,5-bis (*p*-amidinophenoxy)-pentane) is an aromatic diamidine which is useful in the treatment and prophylaxis of pre-CNS trypanosomiasis and leishmaniasis (Sands et al 1985). The pharmaceutical importance of pentamidine has increased recently as a consequence of its activity against HIV-associated *Pneumocystis carinii* infection (Wispelway & Pearson 1991).

Pentamidine can compromise certain functions of neutrophilic granulocytes, at least in-vitro. At concentrations within the therapeutic range after administration of a standard parenteral dose of the drug in-vivo, there is a depression in the candidacidal capacity of stimulated neutrophilic granulocytes (Arnott & Hay 1989a), a phenomenon likely to result from a drug-induced depression of components of the respiratory burst of such cells (Arnott & Hay 1989b); the latter may, in turn, result from interference with the action of the membrane-associated NADPH-oxidase system of these phagocytic cells (Arnott & Hay 1990; Arnott et al 1991).

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Degranulation is one of the major functions of neutrophilic granulocytes; the process results in the release of enzymes which are involved in oxygen-independent killing of microbes (Spitznagel & Schafer 1985).

The primary aim of the present study was to determine in-vitro whether therapeutic concentrations of pentamidine would interfere with degranulation of human neutrophilic granulocytes using the enzymes lysozyme, β-glucuronidase and myeloperoxidase as markers of the process. Activities of the cytosolic enzymes lactate dehydrogenase and glucose-6-phosphate dehydrogenase were also determined after exposure to pentamidine.

Materials and methods

Isolation of neutrophilic granulocytes. Human neutrophilic granulocytes from five normal, healthy adult male donors were separated from heparinized venous blood by sedimentation for 45 min at 37°C with Plasmagel (Universal Biologicals Ltd) in a Plasmagel-to-blood ratio of 1:4 and processed according to the method of Babior & Cohen (1981). Only cell preparations with a cell viability and purity of ⩾98%, as determined by trypan blue exclusion and May–Grünwald–Giemsa staining, respectively, were used in the assays.

Degranulation. Neutrophilic granulocytes ($0·5–2·0 \times 10^7$) were preincubated with pentamidine for 30 min at 37°C. Drug concentrations of 0·3, 0·7, 1·1 and 1·5 mg L^{-1}, calculated in

terms of the isethionate salt (Arnott et al 1988) were used; these represented values within the range of plasma concentrations of the drug expected after parenteral administration of a standard 4 mg kg^{-1} dose in-vivo (Waalkes & DeVita 1971). Controls comprised cell preparations untreated with pentamidine.

Cell preparations were mixed with zymosan and the inhibitor of microfilament assembly, cytochalasin B (Goldstein et al 1975), then shaken at 37°C for 30 min, the reaction being terminated by placing the mixture on wet ice and centrifuging at 280 *g* for 10 min.

The supernatant was assayed for enzymes using methods described by Babior & Cohen (1981). Lysozyme activity was determined turbimetrically using the rate of change in light-scattering induced by degradation of cell walls of *Micrococcus lysodeikticus*. β-Glucuronidase activity was detected by release of *p*-nitrophenol from the hydrolysis of *p*-nitrophenyl β-D-glucuronide. Myeloperoxidase activity estimation used hydrogen peroxide to oxidize *o*-dianisidine to a coloured product. The lactate dehydrogenase (LDH) assay measured NADH consumption. Glucose-6-phosphate dehydrogenase (G-6-PD) was assayed as NADPH formed by enzymatic reduction of NADP by glucose-6-phosphate. Protein determinations for lysozyme and G-6-PD assays were performed using a standard assay method (Lowry et al 1951).

Statistical analysis. Twenty replicates for each enzyme were performed for each concentration of drug and appropriate controls. Data from the assays were compared by analysis of variance (SPSS Inc. 1986). A posteriori comparisons (Scheffé 1953) were used to compare which variable or variables differed from other variables.

Results

Table 1 shows the data obtained from the enzyme assays. The results of these experiments were expressed as the mean and standard deviation of the net release of each enzyme from 20 replicates under test conditions. Statistical comparisons revealed that there were no significant differences, relative to non-drug treated controls, in activity of lysozyme, β-glucuronidase and myeloperoxidase, at any of the four concentrations of pentamidine.

LDH and G-6-PD, however, showed differences in activity ($P < 0.001$ and $P < 0.01$, respectively) when compared with non-drug-treated controls. With the two dehydrogenases, there was no significant difference in activity between drug-treated and untreated samples at a pentamidine concentration of 0·3 mg L^{-1}. Concentrations of 0·7, 1·1 and 1·5 mg L^{-1}, however, induced significant decreases in activity of both enzymes; this decrease was not dose related, the effect being consistent at all three drug concentrations, for both enzymes.

Effect of donor. A series of analyses of covariance, with age of donor as covariant, indicated that the observed depression in LDH and G-6-PD activity was independent of this parameter.

Discussion

The results obtained from the present study indicated that at certain concentrations within the therapeutic range, which might be expected after parenteral administration of a standard 4 mg kg^{-1} dose of pentamidine isethionate, there was no detectable drug-associated effect on neutrophil degranulation after zymosan activation, as assayed by secretion of azurophil granule enzymes myeloperoxidase and β-glucuronidase, or the specific azurophilic granule marker lysozyme. However, significant decreases in the activity of the cytosolic enzymes LDH and G-6-PD were detected at concentrations of 0·7, 1·1 and 1·5 mg L^{-1}.

Unlike previous studies on the effect of pentamidine on neutrophilic granulocyte function (Arnott & Hay 1989a, b, 1990), the depression in enzyme activity was not dose related; no statistically significant differences were discerned at any of the three concentrations of drug which induced compromised activity, although there was an apparent dose-related trend of decreased enzyme activity.

As with previous studies, which indicated that pentamidine suppressed candidacidal activity (Arnott & Hay 1989a), certain components of the respiratory burst (Arnott & Hay 1989b) and NADPH-dependent oxidase activity (Arnott & Hay 1990) of stimulated neutrophilic granulocytes, the recorded depression in activity of LDH and G-6-PD was not the consequence of a generalized toxic effect of pentamidine on the cells; cell viability, as assessed by trypan blue exclusion, was unaffected at any of the drug concentrations used. It should be noted, however, that release of LDH or G-6-PD into the extracellular medium can be used as an indicator of cell disintegration or lysis (Babior & Cohen 1981) and in one study, rat hepatocytes incubated with pentamidine produced leakage of LDH without concurrent interference with cell viability (Sippel & Estler 1990). However, the findings from the present study cannot be explained in terms of cell lysis, since in the case of both enzymes the activity was reduced. These observations are thus strongly suggestive that pentamidine selectively reduced the intracellular activity of LDH and G-6-PD. The direct effect of pentamidine on activity of LDH and G-6-PD remains to be determined.

LDH and G-6-PD are involved in glucose metabolism. LDH is a cytosolic enzyme which acts under aerobic conditions when cells are undergoing very high rates of glycolysis; NADH generated in glycolysis cannot be reoxidized in the mitochondrion, and NADH must be used to drive the reduction of pyruvate to maintain homeostasis; the reaction is catalysed by LDH. In the mouse, pentamidine treatment was associated with

Table 1. Means and standard deviations of net effect[a] of pentamidine isethionate on activity of lysozyme, β-glucuronidase, myeloperoxidase, lactate dehydrogenase and glucose-6-phosphate dehydrogenase.

Drug (mg L^{-1})	Lysozyme (50 μg equivalents (mg protein)$^{-1}$)	β-Glucuronidase (nmol min^{-1})	Myeloperoxidase (nmol min^{-1})	Lactate dehydrogenase (nmol lactate min^{-1})	Glucose-6-phosphate dehydrogenase (μmol min (mg protein)$^{-1}$)
0·3	39·38 ± 12·06	0·09 ± 0·04	408·48 ± 29·13	40·06 ± 4·09	0·20 ± 0·07
0·7	40·92 ± 8·80	0·10 ± 0·03	407·82 ± 28·03	37·72 ± 3·70*	0·18 ± 0·06*
1·1	41·21 ± 8·45	0·10 ± 0·03	407·13 ± 31·09	36·55 ± 4·00*	0·15 ± 0·05*
1·5	41·25 ± 8·31	0·12 ± 0·07	403·39 ± 32·31	34·63 ± 3·18*	0·13 ± 0·06*
Control[b]	41·32 ± 8·58	0·09 ± 0·03	404·01 ± 40·78	40·12 ± 3·83	0·20 ± 0·07

[a] Computed values based on 20 replicates. [b] Cell preparations untreated with pentamidine. *$P < 0.05$ compared with other values.

an initial increase in blood glucose and fatty acid, followed by increased hepatic triglyceride and glycogen contents accompanied by decreases in liver pyruvate, ATP and lactate (Sippel et al 1991). These latter effects were interpreted as a drug-induced, transient inhibition of lipolysis and glycogenolysis with a longer-lasting inhibition of energy producing carbohydrate metabolism. However, in view of the findings from the present study, the latter may, at least in part, have been the consequence of pentamidine-induced compromise of LDH activity.

G-6-PD, a soluble enzyme in the cytosol, is the rate-limiting first-step enzyme of the pentose-phosphate pathway (PPP), catalysing the oxidation of glucose-6-phosphate to 6-phosphoglucono-δ-lactone. G-6-PD serves as an important source of NADPH and of ribose and deoxyribose for nucleic acid synthesis.

Pentamidine induces a dose-dependent reduction of PPP activity (Arnott & Hay 1989b). Furthermore, the drug has a deleterious effect on the cofactors NADPH and FAD in-vitro (Makulu & Waalkes 1975), albeit at very high concentrations. Thus, the reduction in ability of pentamidine-treated neutrophilic granulocytes to kill *Candida albicans* spores may result from a drug-induced compromise of the NADPH-oxidase system (Arnott & Hay 1990), which is directly or indirectly associated with G-6-PD dysfunction which induces a reduction in activity of the PPP (Arnott & Hay 1989b).

Further studies are required to establish if the effects of pentamidine on degranulation of human neutrophilic granulocytes are stimulus specific. For example, is enzyme release triggered by soluble agonists such as FMLP, leukotriene B4, C5a and PAF or particulate stimuli, such as aggregated IgG? It also remains to be determined if true degranulation of neutrophilic granulocytes is affected by pentamidine.

However, findings from the present study taken with previous data concerned with the effects of pentamidine on human neutrophilic granulocytes suggest that biochemical processes in these cells, which involve cofactors, especially NADP(H) and NAD(H), may be compromised by the drug, leading to depression in killing capacity of these phagocytic cells.

Interference with the activity of these biochemical processes by pentamidine may also explain the action of the drug on trypanosomids and *P. carinii*, where there is thought to be an effect on RNA polymerase and ribosomal function; it also provides a tentative explanation for killing of non-replicating cells by the drug (Sands et al 1985).

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