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Different effects of acute and chronic diabetes mellitus on hepatic drug metabolism in the rat

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The effect of diabetes mellitus on drug metabolism in the rat has been known for some time [1–3] and has been shown in spontaneously diabetic animals as well as those with chemically-induced diabetes [4] indicating that the effects seen are related to the diabetes and are not a direct effect of the drug on the liver. The effects reported are diverse and often conflicting, with some workers showing effects only on 'androgen-dependent' pathways in the male [1, 2] and others presenting evidence for a different effect including changes in non-sex-dependent enzyme activities in the female [5]. The effects seem to be related to the substrate under study [4] but may also be related to the different treatment schedules used in the investigations as some workers use acutely diabetic animals (3–4 days after treatment) while others use animals made diabetic 12–20 days earlier. It was decided, therefore, to investigate whether there was a difference in the effects of acute and chronic diabetes mellitus on hepatic drug metabolism in the rat.

Induction of diabetes by streptozotocin (STZ) was chosen for this work as the drug has previously been shown to be a selective B-cell toxin [6] although there are some indications that it may have a direct toxic effect on the liver as well [7]. As discussed above, however, the effect of spontaneous diabetes on drug metabolism is similar to that of STZ and, thus, the STZ would appear to be exerting its effect by causing diabetes.

The sex-dependent nature of the effects of diabetes can only be studied with substrates whose metabolism is sex-differentiated. Lignocaine, imipramine and diazepam all exhibit sex-dependent metabolism in the rat [8, 9] and have been used in the investigations of hormonal control of drug metabolism previously [10, 11]. These drugs should, therefore, prove good models to study the effects of diabetes on hepatic drug metabolism.

The question to be answered in this study was, therefore, is there a difference between the effects of acute and chronic STZ-induced diabetes mellitus on hepatic drug metabolism in the rat?

Materials and methods

Chemicals. Streptozotocin, sodium isocitrate and isocitric dehydrogenase were obtained from Sigma Chemical Co. (London, U.K.). $NADP^+$ was supplied by Boehringer Mannheim Co. (Lewes, U.K.). [2-^{14}C]-diazepam and [methylene-^{14}C] imipramine were obtained from Amersham International p.l.c. and [carbonyl-^{14}C]-lidocaine from New England Nuclear (Southampton, U.K.). Unlabelled lignocaine, diazepam and imipramine were gifts from Astra läkemedel (Stockholm, Sweden), Roche Products (Welwyn Garden City) and Ciba-Giegy AG (Basel, Switzerland), respectively. All other chemicals were of the highest purity available commercially.

Animals. Wistar rats with body weights of 300–350 g (male) or 250–275 g (female) at the beginning of the experiment were used throughout the study. The animals were housed in light- and temperature-controlled conditions (lights on 0800–2000; 19 ± 1°) and allowed free access to food and water. Diabetic animals were kept in wire-bottomed cages due to the excess urine production of these animals.

Diabetes was induced by a single i.v. (tail vein) injection of streptozotocin (60 mg/kg; 180 mg/ml in distilled water) given under halothane/N_2O anaesthesia. The solution of STZ was made up immediately prior to use as the drug is very labile in solution. Control animals were injected under anaesthesia with vehicle only. The animals were left for 3 (acute) or 20 days (chronic) before sacrifice.

Preparation of serum and microsomes. The animals were killed by CO_2-asphyxiation and cervical dislocation. Blood was collected from the cut neck vessels and allowed to clot at 4°. The clotted blood was centrifuged at 2500 *g* in a Damon/IEC DPR-6000 refrigerated centrifuge for 20 min to prepare serum. The serum was stored at −20° until required for assay of glucose. The liver was quickly excised and washed in ice-cold 0.25 M sucrose to remove excess blood and subsequently homogenised in 4 volumes of the same buffer using a Potter-Elvehjem homogeniser with Teflon pestle. Microsomes were prepared from the homogenate by the Ca^{2+}-precipitation technique of Cinti *et al.* [12].

Enzyme assays. The microsomal metabolism of lignocaine, imipramine and diazepam was measured as previously described [8, 9]. The microsomes were incubated at 37° in a shaking waterbath with ^{14}C-labelled substrate in the presence of an NADPH-regenerating system consisting of $NADP^+$ (1.3 mM), sodium isocitrate (5 mM), isocitric dehydrogenase (30 μg/ml) and manganese chloride (0.3 mM). The metabolites and unchanged substrate were extracted into organic solvent, separated by thin-layer chromatography and located by autoradiography. The metabolites were quantitated by liquid scintillation counting of the radioactive bands and the enzyme activities calculated by a custom-made computer program.

Other assays. Serum glucose concentration was measured using a kit from the Sigma Chemical Co. (London, U.K.) based on the glucose oxidase method.

Microsomal protein concentration was assayed by the method of Lowry *et al.* [13] using bovine serum albumin as standard.

Statistics. Group means, variances and standard deviations were calculated and compared using Student's *t*-test or, where appropriate, Duncan's Multiple Range Test. The level of significance was set at $P < 0.05$.

Results and discussion

The results of experiment 1, where the effect of acutely induced diabetes on microsomal phase I metabolism in male and female rats was compared, are shown in Table 1.

It is seen that the diazepam 3-hydroxylation and N-demethylation, lignocaine N-deethylation and imipramine N-demethylation and N-oxidation, which are all sex-dependent enzyme activities, are markedly reduced in the male but unaffected in the female. It is also noted that lignocaine 3-hydroxylase and imipramine 2-hydroxylase activities are unaffected in either sex—these are non-sex-differentiated enzymes [8]. These results confirm the data of Kato *et al.* [1] and indicate that lignocaine, imipramine and diazepam are suitable substrates for investigating the effects of diabetes on hepatic drug metabolism. The differential effect of the disease on male and female animals is not due to the female not responding to the STZ treatment as the increase in serum glucose was similar in both sexes.

In experiment 2 the effects of acute and chronic diabetes in the male on lignocaine, imipramine and diazepam metab-

Table 1. Effect of STZ-induced diabetes on microsomal metabolism of diazepam, lignocaine and imipramine in male and female rats

Parameter	Control♂*	Diabetic♂	Control♀	Diabetic♀
Diazepam				
3-OHase†	92.0 ± 27.1	52.6 ± 7.5‡	30.1 ± 8.5	23.9 ± 2.5
N-Demethylation	72.6 ± 11.0	37.4 ± 9.4‡	31.3 ± 9.0	32.8 ± 13.9
Lignocaine				
3-OHase	35.0 ± 5.9	31.1 ± 1.6	24.2 ± 2.9	24.9 ± 7.1
N-deethylation	317 ± 118	182 ± 65‡	44 ± 14	54 ± 12
Imipramine				
N-oxidase	70 ± 25	35 ± 6‡	42 ± 8	44 ± 13
N-demethylase	810 ± 130	250 ± 51‡	186 ± 11	179 ± 26
2-OHase	48 ± 8	30 ± 9	92 ± 11	97 ± 8
Serum glucose§	8.7 ± 0.7	32.1 ± 2.5‡	7.7 ± 0.2	29.0 ± 2.8‡

* 5 animals in each group; results expressed as mean ± 1 S.D.
† All enzyme activities expressed as pmoles product formed $min^{-1}mg\ protein^{-1}$.
‡ Significantly different from the relevant control; $P < 0.05$.
§ Expressed as mM.

Table 2. Effect of acute (3 day) and chronic (20 day) diabetes mellitus on microsomal drug metabolism in the male rat

Parameter	Acute* control	Acute diabetic	Chronic control	Chronic diabetic
Diazepam				
3-OHase†	184 ± 25	44 ± 13‡	122 ± 24	182 ± 61
N-demethylation	179 ± 15	47 ± 17‡	102 ± 10	90 ± 20
Lignocaine				
3-OHase	28.0 ± 7.9	8.1 ± 1.9‡	15.1 ± 2.2	11.1 ± 7.9
N-deethylase	149 ± 21	19 ± 1‡	151 ± 32	63 ± 19‡
Imipramine				
N-oxidase	56 ± 9	28 ± 12‡	68 ± 24	64 ± 20
N-demethylase	1092 ± 272	276 ± 36‡	924 ± 208	516 ± 164
2-OHase	48 ± 19	40 ± 21	42 ± 6	29 ± 8
Serum glucose§	10.4 ± 2.3	24.3 ± 7.4‡	10.0 ± 0.8	35.5 ± 2.0‡

* 5 animals in each group; results expressed as mean ± 1 S.D.
† All enzyme activities expressed as pmoles product formed $min^{-1}mg\ protein^{-1}$.
‡ Significantly different from the relevant control; $P < 0.05$.
§ Expressed as mM.

olism were investigated. The results are presented in Table 2.

It is seen that acute diabetes had a marked effect on all enzyme activities measured except the imipramine 2-hydroxylase. The finding of an effect on lignocaine 3-hydroxylase in this experiment is unexpected and no explanation can be put forward to explain this result. All other effects were similar to those seen in experiment 1. Chronic diabetes on the other hand gave somewhat different results with no effect being seen on diazepam and imipramine metabolism and only lignocaine N-deethylase showing a significant reduction. The overall affect of chronic diabetes is, thus, much less than that seen in the acute phase. Again similar raised serum glucose levels were seen in acute and chronic diabetes and, thus, a change in the severity of diabetes cannot be put forward as an explanation of the differences in the effects noted.

It can thus be concluded that STZ-induced diabetes mellitus does, indeed, have a different effect on hepatic phase I drug metabolism in the rat in the acute and chronic phase. The effect can be described as sex-dependent and transient. This differential effect of acute and chronic diabetes could be part of the explanation of the discrepancies in effects noted by various workers and it may also, as an added advantage, give some insight into the reasons behind the sex-dependent nature of the effect of diabetes on drug metabolism. The biochemical explanation for the effect of diabetes on drug metabolism may be found by an investigation of other transient effects of the disease. This line of enquiry may even lead to a full explanation of the sex differences in drug metabolism in the rat which has so far been elusive [14].

Further work is in progress to investigate the mechanism of action of STZ-induced diabetes on hepatic drug metabolism in the rat.

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Production of a monoclonal antibody-mitomycin C conjugate, utilizing dextran T-40, and its biological activity

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Attempts to bind anticancer agents to antibodies have increased because of the hydbridoma technique developed by Köhler and Milstein [1] which permits the production of large amounts of monoclonal antibodies. Theoretically, the selective cytotoxicity of anticancer agents should be enhanced by conjugation to antibodies raised against antigens on the surface of tumor cells. For this reason, methods of linking anticancer drugs covalently to antibodies have been investigated [2, 3]. In our laboratory, a hybridoma cell line which produces an anti-HLA* IgG_1 monoclonal antibody (H-1) was established, and H-1 was shown not to exhibit cytotoxic activity *in vitro*. The antibody (H-1) seemed to be ideal for assessing the activity of linked drugs and the antigen-targeting potential of anticancer drug (H-1) conjugates [4].

Mitomycin (MMC), an antibiotic isolated from *Streptomyces caespitosus* by Wakaki *et al.* [5], has potent anticancer activity; however, its clinical use is limited by its detrimental effects on normal tissues. MMC-antibody conjugates were first prepared by Suzuki *et al.* [6], employing the cyanogen bromide method.

In this report, we conjugated MMC to H-1 IgG_1 antibody with the use of dextran T-40 as a multivalent carrier, and studied the cytotoxic activity of the conjugate against cells bearing or lacking HLA *in vitro*.

Materials and methods. MMC was obtained from the Kyowa Hakko Co., Ltd., Tokyo. Dextran T-40 was purchased from Pharmacia Fine Chemicals, Sweden, and sodium periodate and sodium borohydride were from the Sigma Chemical Co., U.S.A. A null cell line (NALL-1) [7], which has HLA, was derived from human acute lymphoblastic leukemia cells, and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. A mouse myeloma cell line, P3-NS1/1-Ag4-1 (NS-1) [8], which does not have HLA, was used as a control. The hybridoma cells secreting H-1 antibody [4] were grown in the peritoneal cavity of BALB/c mice, and the ascitic fluid from several mice was pooled. Control IgG was obtained similarly using NS-1 cells. H-1 and control IgG were purified from the ascitic fluid pool by affinity chromatography on Protein-A Sepharose CL-4B [9, 10].

Dextran T-40 (molecular weight: 4×10^4) was oxidized to polyaldehyde-dextran (PAD) by the following Malaprade reaction [11, 12]. Dextran T-40 (1.0 g) was mixed with sodium periodate (0.33 g) in 200 ml of distilled water, and stirred for 1 hr at room temperature. The resultant mixture was concentrated using a Collodion bag (cut-off level: 2.5×10^4; Sartorius, Germany), applied to a Sephadex G-25 column equilibrated with distilled water, and lyophilized. Sixty milligrams PAD was incubated with 20 mg H-1 in 10 ml of 0.15 M phosphate-buffered saline (PBS), pH 7.2, for 24 hr at 4°, after which 12 mg MMC in 5 ml PBS was added. The solution was stirred for another 24 hr. The Schiff bases thus formed were reduced by 2 hr of incubation with 0.3 ml of sodium borohydride solution (10 mg sodium borohydride in 10 ml PBS) [13]. The mixture was then applied to a Sephadex G-200 column (2.6×100 cm) equilibrated with PBS. The optical density of the fraction was measured at 280 nm, and the two major peaks were lyophilized.

The antimicrobial activity [MMC equivalent (μg/ml)] of the conjugate and of free MMC was assayed by a cup method using *Escherichia coli* ATCC 11303 agar plates and measuring the zone of growth inhibition [14]. The degree of MMC substitution of the conjugate was estimated by the absorbance at 363 nm, assuming an $E^{1\%}_{1cm,623}$. The IgG concentration was measured by the Bio-Rad Protein Assay (Bio-Rad Laboratories, U.S.A.) [15]. MMC activity (%) of the conjugate was calculated as follows: MMC activity (%) = MMC equivalent determined by the cup method/MMC substitution determined spectrophotometrically $\times$ 100.

NALL-1 cells were incubated with H-1 or MMC-(H-1) for 30 min at 4° and then stained with fluorescein isothiocyanate (FITC)-conjugated goat antiserum to mouse IgG (E. Y Laboratories, U.S.A.). Membrane staining of the cells was examined by fluorescence microscopy, as was the reactivity of the MMC-(H-1) conjugate to NS-1 cells.

The cytotoxic activity of the conjugate was measured against the HLA-bearing cell line, NALL-1, and the HLA-lacking cell line, NS-1. Cells (3×10^6), grown to the logarithmic phase, were incubated in 1 ml medium containing the test substances for 30 min at 37% and washed two times with fresh medium and cultured further for 3 days in 5 ml medium. Viable cells were measured by the trypan blue dye exclusion test. Data were analyzed for statistical significance according to Student's *t*-test.

Whether MMC was released from MMC-(H-1) conjugate was examined. One milligram of MMC-(H-1) was sus-

* HLA, antigens of the major histocompatibility antigen system A, B and C.