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STUDIES ON THE REACTIVITY OF ACYL GLUCURONIDES—VIII. GENERATION OF AN ANTISERUM FOR THE DETECTION OF DIFLUNISAL-MODIFIED PROTEINS IN DIFLUNISAL-DOSED RATS*

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Abstract—Acyl glucuronide metabolites of carboxylic drugs such as the salicylate derivative diflunisal (DF) have been shown to react with proteins to produce covalent adducts. To aid in the study of the formation and distribution of these adducts in both humans and rats, we raised an antiserum against human serum albumin modified by covalent attachment of DF *via* an amide bond, using a carbodiimide reagent. This antiserum had wide reactivity, reacting with all types of DF-modified proteins tested and with free DF (albeit at a lower affinity). It did not cross-react with other salicylates or other non-steroidal anti-inflammatory drugs. The antiserum has been used in immunoblotting to detect proteins covalently modified by DF in the plasma and livers of rats treated with the drug for 7 days. Although some cross-reactivity was apparent on the blots, a series of DF-modified proteins was found in cytosolic, mitochondrial and mixed membrane fractions of hepatocytes, with molecular weights ranging from 28 to 130 kDa.

Key words: glucuronidation; diflunisal; liver; antibodies; immunoblotting

Conjugation with glucuronic acid is usually a major route of metabolism for drugs bearing carboxylic acid groups. These acyl glucuronide metabolites are intrinsically reactive species *in vivo* and at physiological pH *in vitro*, capable of undergoing hydrolysis, intramolecular rearrangement, and, in the presence of protein, covalent binding reactions [1–3]. Hydrolysis, leading to regeneration of the parent carboxylic drug, occurs during systemic circulation of acyl glucuronides *in vivo*. Catalysts may include hydroxide ion, β-glucuronidases, esterases and serum albumin [2, 3]. Intramolecular rearrangement occurs by hydroxide ion-catalysed migration of the drug moiety away from the biosynthetic 1-*O*-β position, sequentially to the neighbouring 2-, 3- and 4-positions on the glucuronic acid ring [4]. The migrations amongst the isomers so formed are reversible. The isomers (but not the glucuronide itself) can exist in both β- and α-anomeric forms at C-1, through the intermediacy of the open chain form of the glucuronic acid ring [4–6].

Two different mechanisms have been advanced to account for the covalent binding of acidic drugs, via their acyl glucuronide metabolites, to protein. The transacylation mechanism involves displacement of the glucuronic acid moiety by nucleophilic groups such as -SH, -OH and -NH_2 on the protein, leading to direct linkage of the drug to protein by a thioester, ester or amide bond [7, 8]. This pathway should be strongly favoured by the glucuronide as compared to its isomers [9]. The alternative imine mechanism [10, 11] is analogous to the non-enzymic glycation of serum albumin, and *requires* acyl migration to form the isomers before interaction with the protein. The open chain form of each isomer (existing as a transient intermediate between the β- and α-anomers) contains a reactive aldehyde group which can condense with lysine ε-amino groups to form an imine. Subsequent Amadori rearrangement could then lead to a more stable ketoamine. In this mechanism, the glucuronic acid moiety is retained in the adduct, with the drug still linked to it via an ester group. It seems certain at this stage from both indirect [e.g. 12, 13] and direct structural [14, 15] evidence that both mechanisms are operative.

Of the three manifestations of acyl glucuronide reactivity, there has been increasing interest in adduct formation, since the covalent attachment of foreign compounds to endogenous macromolecules

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‖ Abbreviations: DF, diflunisal; DAG, diflunisal acyl glucuronide; *iso*-DAG, mixture of 2-, 3- and 4-*O*-acyl isomers of diflunisal acyl glucuronide formed by acyl migration; HSA, human serum albumin; DF–HSA-I, DF coupled by its carboxyl function to HSA via an amide bond; DF–HSA-II, HSA modified by incubation with a mixture of DAG and *iso*-DAG; KLH, keyhole limpet haemocyanin; DF–KLH, KLH modified by the hemi-succinate derivative of DF; TBS, Tris-buffered saline; NSAID, non-steroidal anti-inflammatory drug.

in vivo has been associated with toxic and/or autoimmune consequences [2, 16]. To date, most attention has focused on the formation of covalent adducts with plasma protein, notably HSA‖ *in vitro* and *in vivo* [2]. Recently, however, adducts of clofibric acid [17] and diclofenac [18, 19] with rodent liver protein have been described. We have shown previously that DF, a salicylate derivative which forms a reactive acyl glucuronide (DAG) [5, 9] as well as stable phenolic glucuronide [5, 20] and sulphate [21] conjugates, forms covalent adducts with plasma protein [22], liver, kidney, intestine, skeletal muscle [23] and urinary bladder [24] of rats dosed with the drug, and with human plasma proteins *in vitro* and *in vivo* [22, 25]. DF, like most other acidic NSAIDs, has been associated with hypersensitivity responses in humans [26]. The present study concerns the generation and partial characterisation of an antiserum against DF, and the utility of this antiserum in detecting DF-modified proteins in plasma and liver fractions from rats dosed with the drug.

MATERIALS AND METHODS

Materials and animals

DF was a gift from Merck, Sharp and Dohme (Sydney, Australia). Ibuprofen and flurbiprofen (Boots, Nottingham, U.K.), naproxen (Syntex, Sydney, Australia), and paracetamol (Parke-Davis, Sydney, Australia) were also gifts. Acetylsalicylic acid and 5-sulphosalicylic acid were purchased from BDH Chemicals (Poole, U.K.). Succinyl dichloride, 1 - cyclohexyl - 3(2 - morpholinoethyl)carbodiimide metho-*p*-toluenesulphonate, 5-aminosalicylic acid, essentially fatty acid-free HSA (product A1887), essentially globulin-free HSA (product A8763), Freund's complete and incomplete adjuvant, equine myoglobin, KLH and Sigma 104 phosphatase substrate tablets were obtained from Sigma-Aldrich (Sydney, Australia). Isobutylchloroformate was purchased from Tokyo Kasei (Tokyo, Japan) and *N*-hydroxysulphosuccinimide from Fluka (Buchs, Switzerland). Microtitre plates were obtained from A/S Nunc (Roskilde, Denmark). Biotinylated anti-rabbit immunoglobulin antibodies, biotin/streptavidin/alkaline phosphatase complex, biotin/streptavidin/horseradish peroxidase complex, enhanced chemiluminescence (ECL) reagents, nitrocellulose (ECL grade) and Hyperfilm-ECL were purchased from Amersham (Amersham, U.K.). All other reagents were of at least analytical grade purity. Rabbits (New Zealand white) were obtained from the Central Animal Breeding House of The University of Queensland. Male Sprague–Dawley-derived rats were obtained from The University of Queensland Medical Faculty Animal House. Experiments were approved by the University's Animal Experimentation Ethics Committee.

Preparation of the immunogen DF–HSA-I—Coupling of DF to HSA using the carbodiimide method

This method was adapted from that used by Hayase *et al.* [27] to couple caproyl pyrraline to bovine serum albumin. DF (78 mg) and HSA (70 mg, essentially globulin-free) were dissolved in water (20 mL) and the pH was adjusted to 7.0 using 0.5 M sodium hydroxide solution. A solution of 14 mg *N*-hydroxysulphosuccinimide in 1.5 mL water was added to the mixture followed by 423 mg 1-cyclohexyl-3(2-morpholinoethyl)carbodiimide metho-*p*-enesulphonate in 4 mL water. The reaction was allowed to proceed for 15 min at room temperature. The mixture was then dialysed against 150 vol. of PBS (3 changes over 48 hr) and the dialysate stored in small aliquots at −20°. This procedure led to the coupling of *ca.* 6.0 μg DF/mg HSA, with the DF being directly linked to protein by an amide group (Fig. 1a, structure based on previous reports of this coupling chemistry [27]).

Production of antisera against DF–HSA-I

A set of multi-site s.c. injections consisting of 1.5 mg DF-modified protein emulsified in Freund's complete adjuvant was administered to a rabbit. This was followed by three i.m. injections of 600 μg modified protein in Freund's incomplete adjuvant given at 4 week intervals. Finally, two sets of s.c. injections (600 μg modified protein in Freund's incomplete adjuvant) were given at 4 week intervals. One week after the final injection, the rabbits were exsanguinated by cardiac puncture under pentobarbitone anaesthesia. The blood was heparinised and the separated plasma stored at −20°.

Preparation of DF-modified proteins for use in ELISA

DF–HSA-II—Coupling of DF to HSA via DAG and its rearrangment isomers. DAG and a mixture of its rearrangement isomers (*iso*-DAG) were prepared as previously described [20]. A 20 mg/mL solution of HSA (essentially fatty acid-free) in 100 mM sodium phosphate buffer pH 7.4 was incubated with DAG and *iso*-DAG, each at 200 μg DF equivalents/mL, for 12 hr at 37°. Further portions of DAG and *iso*-DAG (150 μg DF equivalents/mL each) were then added and the reaction allowed to continue for another 12 hr. This solution was then dialysed against 150 vol. of PBS (three changes over 48 hr). The final dialysate was filtered to remove any precipitate formed during dialysis, and stored in small aliquots at −20°. Based on knowledge to date (see Introduction), the structures of the DF–HSA-II adducts will comprise DF directly linked to protein via an ester, thioester or amide bond (Fig. 1b) and/or DF linked via an ester group to the 4-, 3- or 2-positions of the glucuronic acid moiety which in turn is linked to protein via an imine or ketoamine bond (Fig. 1c illustrates the 4-position/imine structure).

DF–KLH—Synthesis and coupling of DF-hemisuccinate to KLH. This method was based on that described by Wurzberger *et al.* [28] to derivatise haloperidol. Succinyl dichloride (200 μL) was added to 100 mg of DF dissolved in 12 mL of chloroform and vigorously mixed. The reaction was allowed to proceed for 30 min at room temperature and was terminated by the addition of 6 mL of water. Then, 9 mL of 100 mM sodium phosphate buffer (pH 6.8) was added and the mixture adjusted to pH 6.8 with 0.5 M sodium hydroxide solution. The hemisuccinate derivative was extracted with 45 mL of chloroform which was dried using anhydrous sodium sulphate

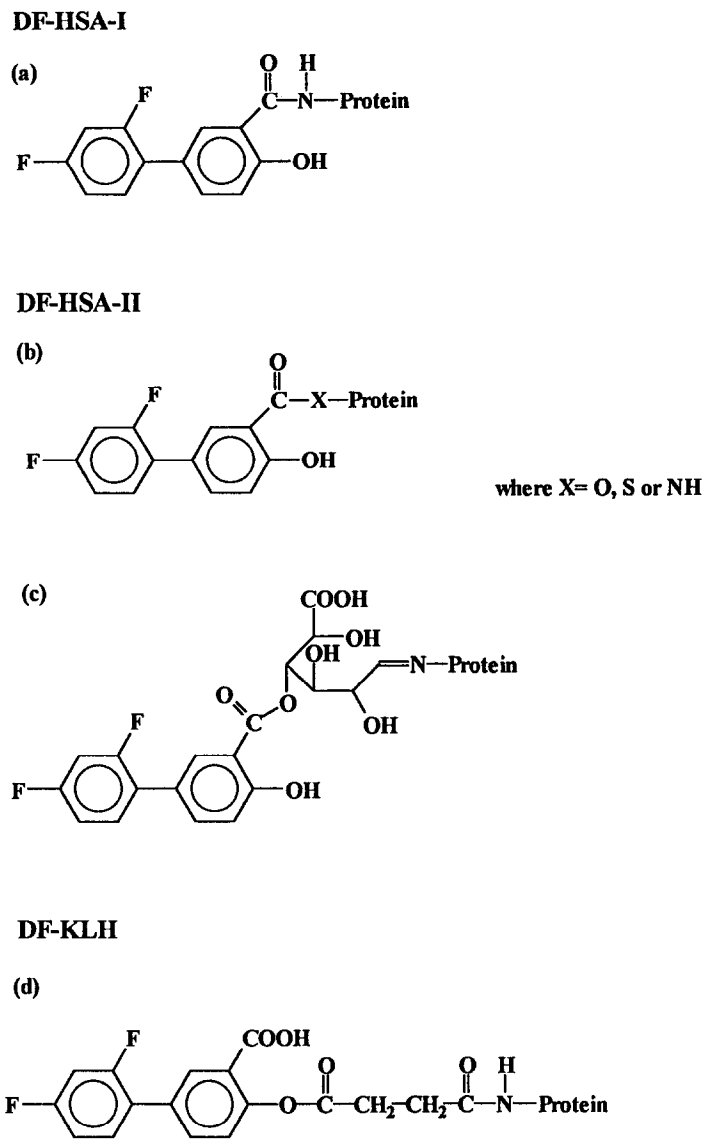

Fig. 1. Proposed chemical structures of DF–protein adducts used for immunisation and ELISA. DF–HSA-I (a): DF linked via an amide group to HSA using a carbodiimide reagent; DF–HSA-II (b): DF linked to HSA by incubation with DAG, i.e. transacylation mechanism; DF–HSA-II (c): DF linked to HSA by incubation with *iso*-DAG, i.e. rearrangement/glycation mechanism, exemplified with the 4-isomer; DF–KLH (d): DF linked to KLH via a hemisuccinate derivative.

and evaporated under nitrogen. The residue was dissolved in 5.3 mL dioxane together with 55 μL of triethylamine and 52 μL isobutylchloroformate and kept at 8° for 20 min. This mixture was added dropwise to 4 mL of 100 mM borate buffer (pH 9) containing 38 mg KLH and 2.7 mL dioxane. The mixture was adjusted to pH 8.5 and incubated at room temperature for 40 min and then overnight at 4°. The reaction mixture was dialysed against 100 vol. of 50% (v/v) aqueous dioxane (two changes over 36 hr), 100 vol. of water (three changes over 24 hr) and 100 vol. of PBS (three changes over 24 hr). The final dialysate was filtered to remove any precipitate which had formed during dialysis and stored in small aliquots at −20°. The structure of the adduct, involving DF linked to protein via a hemisuccinate spacer group (Fig. 1d), is based on previous reports of this coupling chemistry [28].

Human haemolysate and equine myoglobin were also modified by DF using essentially the same methods as described for the production of DF–HSA-I and DF–HSA-II.

Measurement of antiserum reactivity with a range of DF-modified proteins and free drugs

Direct ELISA. An ELISA, adapted from that described by Worrall *et al.* [29] to measure antibody reactivity with acetaldehyde-modified proteins, was

used to determine the reactivity of the antiserum with control KLH and DF–KLH. Microtitre plates were coated with 100 μL of DF–KLH or control KLH (both 50 μg/mL solutions in 0.1 M sodium carbonate/bicarbonate buffer pH 9.0) overnight at 4°. The plates were washed with 50 mM Tris, pH 7.4, containing 145 mM sodium chloride and 0.5% (w/v) casein (TBS-casein) and non-specific binding sites blocked by incubation with saturated casein solution for 1 hr at room temperature. The antiserum was serially diluted in TBS-casein and 100 μL was added to each well and incubated for 1 hr at 37°. After washing with TBS-casein, 100 μL of biotinylated donkey anti-rabbit immunoglobulin, diluted 1:5000 in TBS-casein, was added to each well and incubated for 1 hr at 37°. The plates were then washed and 100 μL of streptavidin/biotin/alkaline phosphatase complex, diluted 1:1000 in TBS-casein, was added. After a 30 min incubation at 37°, the wells were washed as before and 100 μL of *p*-nitrophenyl phosphate (1 mg/mL) in diethanolamine buffer (10 mM diethanolamine, 0.5 mM magnesium chloride, pH 9.5) was added. The reaction was allowed to proceed for 15 min at 37°. The absorbance of each well was read at 405 nm using a Titertek Multiskan plate reader.

Inhibition ELISA. This type of ELISA was essentially the same as that described above except that a single antiserum dilution was used rather than a dilution series. The antiserum was incubated for 30 min at room temperature with various concentrations of DF-modified proteins (HSA, human haemolysate and equine myoglobin) or free drugs (DF, the salicylates aspirin, 5-aminosalicylic acid and 5-sulphosalicylic acid, the arylpropionic acid NSAIDs ibuprofen, flurbiprofen and naproxen, and paracetamol) before being added to the plate. If the antiserum reacted with the DF-modified protein or free drug, it would be unable to bind to the DF-modified protein coating the plate, which would lead to a decreased final absorbance when compared to antiserum preincubated with buffer.

Purification of the antiserum by immunoadsorption

For the inhibition experiments utilising DF-modified HSA, the antiserum was depleted of reactivity with unmodified HSA by immunoadsorption. Antiserum (1 mL) was mixed with 1 mL of buffer A (28 mM sodium chloride, 20 mM Tris–HCl, pH 8.0) and applied to a Biogel P-6DG column. The antiserum was eluted using 3.5 mL of buffer A and applied to a 10 mL DEAE-Affigel blue column pre-equilibrated with buffer A. The column was eluted using buffer A and the eluate monitored for protein content (absorbance at 280 nm). The fractions with the highest protein concentrations were pooled, applied to a column of Affigel-10 matrix modified with HSA and incubated for 2 hr at room temperature. The HSA-modified matrix was prepared by incubating 250 mg of HSA with 10 mL of activated ester gel in 25 mL of buffer (160 mM calcium chloride, 40 mM Hepes pH 7.5) overnight at 4°, followed by washing with PBS to remove any uncoupled protein. The eluate from this column was then assayed for immunoreactivity by direct ELISA.

Administration of DF to rats

Rats were prepared, under methoxyflurane anaesthesia, with a catheter in the right external jugular vein as described previously [30], and then placed unrestrained in metabolism cages. Food and water were available *ad libitum*. After a recovery period of at least 2 hr, dosing with DF was commenced at 50 mg/kg (10 mg DF/mL 0.1 M $NaHCO_3$) i.v. twice daily (about every 12 hr) for 7 days. Rats were killed under anaesthesia by exsanguination via the aorta 6 hr after the last dose and the liver was perfused immediately with PBS via the portal vein. The liver was then excised, snap frozen and stored at −70°. The arterial blood samples were centrifuged and aliquots of plasma stored at −70°. Control rats were killed by exsanguination without prior catheterisation and their livers and plasma samples obtained by the same methods as above.

Liver subcellular fractionation

Livers obtained from control and DF-treated rats were fractionated as described by Touster *et al.* [31]. Briefly, 1 g samples of livers were homogenised in 3 vol. of buffer (20 mM Hepes, pH 8.0, containing 0.25 M sucrose, 1 mM EGTA, 1 mM EDTA and 100 μM phenylmethylsulphonyl fluoride) and centrifuged for 15 min at 1000 *g*. The pellet was rehomogenised in 1 vol. of buffer and again centrifuged. The supernatants were pooled and centrifuged for 10 min at 33,000 *g*, and the pellet (mitochondrial fraction) was resuspended in 1 mL buffer. The 33,000 *g* supernatant was then centrifuged at 78,000 *g* for 40 min. The resultant pellet (mixed membrane fraction) was resuspended in 1 mL buffer. The supernatant contained the cytosolic proteins. This methodology has been optimised to give the best combination of yield, purity (<2% contamination in each fraction) and reproducibility. Although the mixed membrane fraction can be further separated by density gradient centrifugation into plasma membrane, endoplasmic reticulum and other internal membrane fractions, this further purification was not attempted in the present study.

Western blotting of DF-modified proteins

Western blotting of proteins from plasma and subcellular fractions of liver from control and DF-treated rats was carried out essentially as described in Worrall *et al.* [32] except that samples were incubated with buffer at room temperature for 30 min rather than boiled. Briefly, liver and plasma proteins were separated on 10% polyacrylamide gels under denaturing conditions [33]. The gel was then equilibrated with transfer buffer for 20 min and blotted on to nitrocellulose sheets using the method described by Tsang *et al.* [34]. Non-specific binding sites on the nitrocellulose sheets were blocked by incubation with 5% (w/v) low-fat skimmed milk in TBS. Prior to incubation with the blots, antiserum raised against DF-HSA-I was preincubated with 70 mg/mL HSA (fatty acid-free) for 1 hr at room temperature to deplete the antiserum of reactivity with unmodified HSA. The antiserum was then diluted 1 in 200 (v/v) with 1% (w/v) skimmed milk

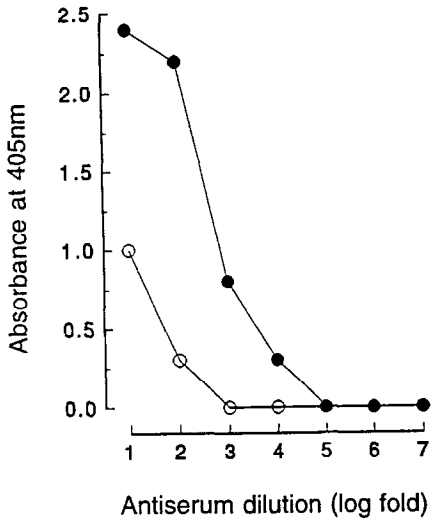

Fig. 2. Immunoreactivity of the antiserum raised against DF–HSA-I with DF–KLH (●) and unmodified KLH (○) as measured by ELISA. Data are presented from the final bleed, with the assay carried out in triplicate (SEM < 10%).

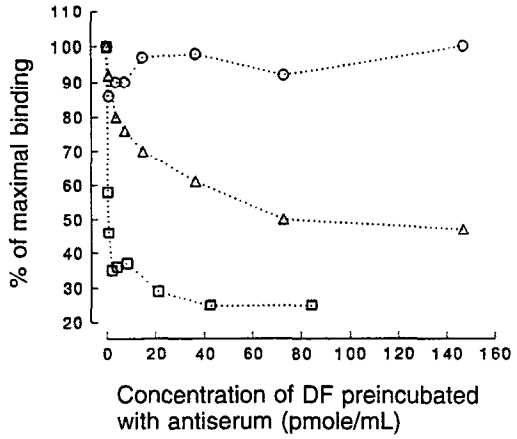

Fig. 3. Inhibition of immunoreactivity of the antiserum raised against DF–HSA-I with DF–KLH by preincubation with human haemolysate (○), human haemolysate modified with DF by incubation with DAG and *iso*-DAG (△) and human haemolysate modified with DF using the carbodiimide method (□). Data are presented as the means of at least three experiments carried out in duplicate (SEM < 10%).

in TBS and incubated with the blots for 1 hr at room temperature. The blots were washed three times for 10 min with 1% skimmed milk and then incubated for 40 min with a 1 in 400 (v/v) dilution of biotinylated donkey anti-rabbit immunoglobulin. After washing as before, streptavidin/biotin/horseradish peroxidase, diluted 1 in 1000 (v/v), was added and the blots were incubated for 30 min at room temperature. Following extensive washing (20 min followed by three washes for 10 min), the blots were rinsed with TBS and the bound antibody visualised by enhanced chemiluminescence using Hyperfilm-ECL.

To test the specificity of the immunodetection of DF adducts, the antiserum was preincubated with DF–HSA-I (at 2.6 nmol covalently bound DF/mL) or an equivalent concentration of unmodified HSA. The antiserum was then diluted to 1 in 500 (v/v) with TBS containing 1% skimmed milk, and the immunodetection procedure continued as described above.

Analyses

For analysis of covalently bound DF, samples of DF-modified proteins were precipitated, exhaustively washed with solvent and base digested, and the DF thus liberated quantified by HPLC as described previously [22], except that ketoprofen was used as internal standard [12]. Proteins were determined according to the method of Lowry *et al.* [35] with HSA (fatty acid-free) as standard. In ELISA, antibody binding to DF-containing epitopes was defined as the difference in absorbance between wells coated with unmodified KLH and DF–KLH.

RESULTS

Approximately 1 week after each injection of the immunogen DF–HSA-I (Fig. 1a), plasma samples from the rabbit were screened for antibody reactivity with DF–KLH (Fig. 1d) by direct ELISA. In each case, immunoreactivity with the DF-modified protein was elevated when compared with unmodified KLH (data for the final bleed is shown in Fig. 2), suggesting the presence of antibodies reactive with modifications derived from DF. To determine reactivity with other forms of DF, the antiserum was incubated with a variety of DF-modified proteins prior to inhibition ELISA. Human haemolysate modified with DF using the carbodiimide method (i.e. the method used to prepare the immunogen, DF–HSA-I) was the most effective in inhibiting the binding of the antiserum to DF–KLH-coated plates (Fig. 3). Haemolysate modified with DF by incubation with DAG and *iso*-DAG was also able to inhibit antibody binding, whereas unmodified haemolysate had no effect. DF-modified HSA (Fig. 4) and equine myoglobin (data not shown) also reduced reactivity with DF–KLH, suggesting that antibodies which were reactive with DF-containing epitopes and which were not carrier protein-specific had been generated.

Once it had been established that this antiserum reacted with a wide range of DF-modified proteins, its reactivity with free DF, DAG and *iso*-DAG, as well as other salicylates and other NSAIDs, was investigated. An inhibition ELISA showed that free DF, DAG and *iso*-DAG inhibited antiserum reactivity with DF–KLH (Fig. 5), albeit at a lower affinity than DF bound to protein. For example, inhibition of binding by *iso*-DAG (Fig. 5) at 1000 pmol DF equivalents/mL (i.e. 10^{-6} M) was ~50%, whereas inhibition of binding by DF–HSA-I (Fig. 4) at 40 pmol DF equivalents/mL was essentially complete. Aspirin, 5-aminosalicylic acid, 5-sulphosalicylic acid, ibuprofen, flurbiprofen, nap-

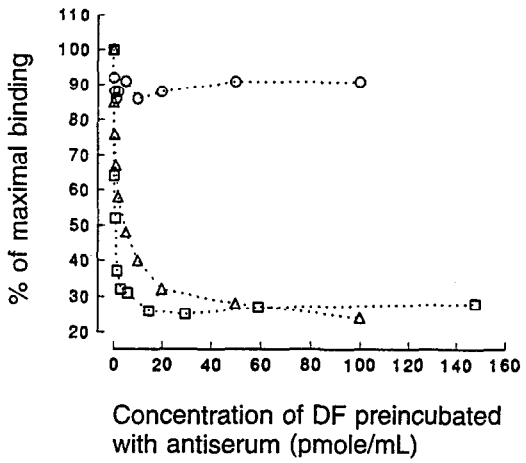

Fig. 4. Inhibition of immunoreactivity of the antiserum raised against DF–HSA-I with DF–KLH by preincubation with HSA (○), DF–HSA-I (□) and DF–HSA-II (△). The antiserum was immunoadsorbed to deplete antibodies reactive with unmodified HSA prior to the experiment. Data are presented as the means of at least three experiments carried out in duplicate (SEM < 10%).

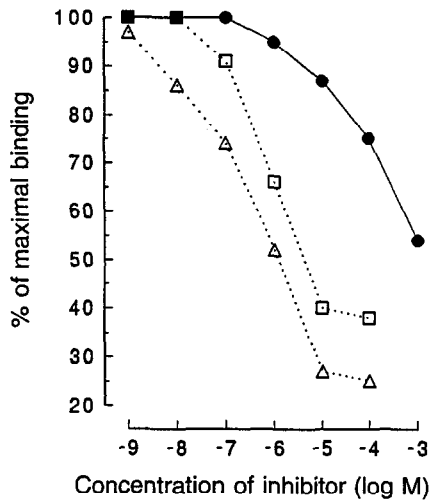

Fig. 5. Inhibition of immunoreactivity of the antiserum raised against DF–HSA-I with DF–KLH by preincubation with DF (●), DAG (△) and *iso*-DAG (□). Data are presented as the means of at least three experiments carried out in duplicate (SEM < 10%).

roxen and paracetamol had no effect on antiserum reactivity.

The antiserum raised against DF–HSA-I thus appeared to be suitable for detection of proteins covalently modified by DF *in vivo*. Rats were dosed with 50 mg DF/kg i.v. twice daily for 7 days, and exsanguinated 6 hr after the last dose. Immunoblotting revealed the presence of a series of DF-modified proteins in plasma and subcellular fractions of livers from these rats (Fig. 6). For the subcellular fractions, the major binding was found in the mitochondrial and mixed membrane fractions. Antiserum reactivity with proteins in cytosol was generally very weak: the blot reproduced in Fig. 6 is that which showed the strongest reactivity. The major modified proteins ranged in molecular weight from 28 to 130 kDa (Table 1). Some reactivity with control proteins was observed (Fig. 6, control). Preincubation of the antiserum with HSA had no effect on reactivity with mitochondrial proteins from DF-treated animals. However, preincubation with DF-HSA-I abolished reactivity with the modified proteins observed in this fraction (Fig. 7), demonstrating a specific interaction with DF-containing epitopes.

DISCUSSION

In this study we describe the generation of an antiserum against DF–HSA-I (i.e. DF coupled by its carboxyl function to HSA *via* an amide bond, Fig. 1a). When tested by ELISA, this antiserum showed higher reactivity with DF–KLH (KLH modified by DF-hemisuccinate, Fig. 1d), than with control KLH (Fig. 2), suggesting that it contained antibodies reactive with DF-containing epitopes. The interaction with DF–KLH could be inhibited by preincubation with human haemolysate modified by DF either using the carbodiimide method or by incubation with DAG and *iso*-DAG (Fig. 3) and by preincubation with free DF (Fig. 5). These data suggest that this antiserum contains antibodies reactive with epitopes present on all of the DF modifications and on free DF. Furthermore, since essentially similar results were obtained using HSA (Fig. 4) and equine myoglobin (data not shown) as carrier proteins, these antibodies appear to react with the DF modification *per se*, irrespective of the carrier protein and mechanism of modification. The antiserum was not reactive with other salicylates, paracetamol or a range of arylpropionate NSAIDs. Therefore, this antiserum was used to detect DF-modified proteins formed in rats dosed with the drug.

In parallel work not presented, we also tried to raise an antiserum against DF–HSA-II (i.e. HSA modified by incubation with DAG and *iso*-DAG), but found that antibodies reactive with DF-containing epitopes were not generated. The reasons for the failure of DF–HSA-II to elicit an immune response are unclear, although the lower extent of DF-modification of the immunogen (*ca.* 20-fold less than that in the DF–HSA-I) may have played a role. Thus, considerably less immobilised DF was injected during the immunisation procedure. Additionally, this type of adduct may be less (or not) stable during antigen processing. Alternatively, it is possible that the particular rabbit used in this study simply had low responsiveness. Whether this result has any implications for the immunogenicity of protein adducts of acidic drugs formed *in vivo* (via acyl glucuronides) is unclear at present, but is the subject of further studies.

Previous studies in our laboratory [23, 24] have demonstrated the formation of covalent adducts of

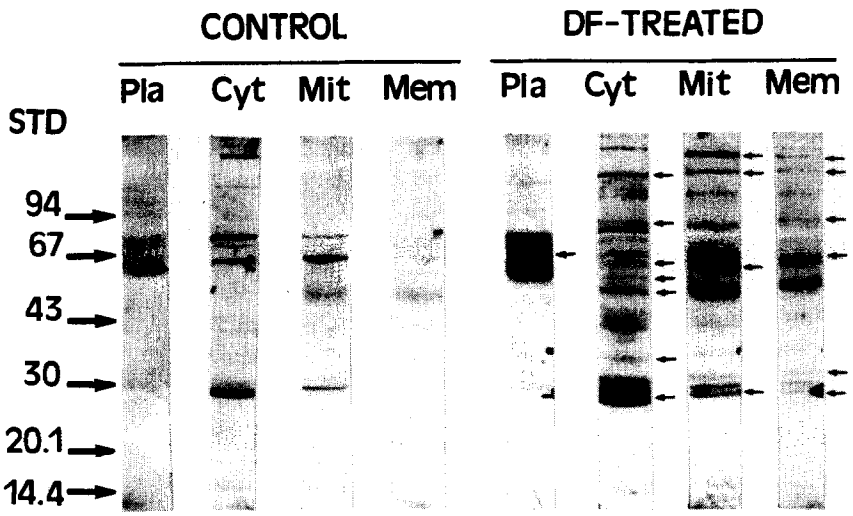

Fig. 6. Western blots showing the reactivity of the DF–HSA-I antiserum with plasma and liver subcellular fractions from control and DF-treated rats. Pla, plasma; Cyt, cytosol; Mit, mitochondria; Mem, mixed membrane. The positions of molecular weight standards are shown under STD (M_r in kDa). The arrows show DF-modified bands not present in controls. The blots for mitochondria and mixed membrane fractions are representative of those obtained; cytosol generally showed little antiserum reactivity, and the blot shown is that exhibiting the strongest reactivity.

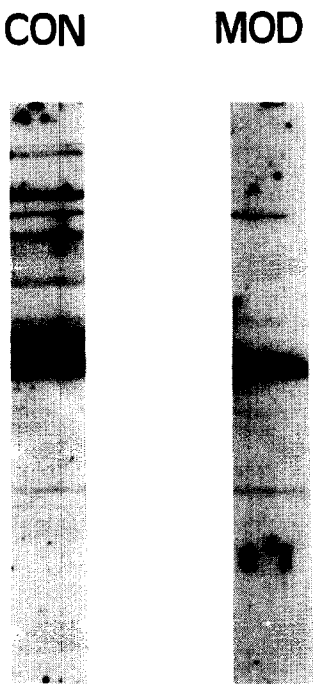

Fig. 7. Western blots showing the inhibition of reactivity of the DF–HSA-I antiserum with liver mitochondria fractions from DF-treated rats caused by preincubation with DF–HSA-I (MOD) as compared with unmodified HSA (CON).

DF with tissues of rats dosed with the drug. In order to characterise further the proteins involved in and the site(s) of adduct formation, we have used the antiserum raised against DF–HSA-I to probe Western blots of proteins from plasma and cytosolic, mitochondrial and mixed membrane fractions of livers from control and DF-treated rats. Albumin appeared to be the major protein modified in plasma, whereas a number of proteins were found to be modified in the subcellular fractions of livers (Table 1). Antiserum reactivity with DF-modifications was strongest in the mitochondrial and mixed membrane fractions, with much less reactivity in cytosol. Several of the modified proteins may be the same as those reported by Pumford *et al.* [18], who detected protein adducts of diclofenac with apparent molecular weights of 50, 70, 110 and 140 kDa in crude homogenates of livers of mice treated with this NSAID. Kretz-Rommel and Boelsterli [19, 36] also reported covalent adducts of diclofenac, with apparent molecular weights of 50, 60, 80 and 126 kDa, in subcellular fractions of cultured rat hepatocytes and in liver homogenates of rats treated with the drug.

The *in vivo* consequences of covalent binding of DF to tissues have not been established, but it has been suggested that such attachment of foreign compounds to endogenous macromolecules may result in immune or toxic responses [2, 16]. DF has been associated with hypersensitivity responses [26] and with cutaneous and fixed drug eruptions [37] and, as a class, the acidic NSAIDs forming acyl glucuronides are associated with a relatively high incidence of toxic or immune responses.

In conclusion, we have raised antibodies against DF covalently bound to HSA. The antiserum reacts

Table 1. Apparent molecular weights of DF-modified proteins in plasma and subcellular fractions of livers from rats dosed with DF for 7 days

Molecular weight (kDa)			
Plasma	Cytosol	Mitochondria	Membrane
—	—	130	130
—	115	115	115
—	85	—	85
67	67	64	67
—	60	—	—
—	55	—	—
—	37	—	32
—	28	28	28

strongly with a variety of proteins chemically modified by DF, and has been used for the detection of DF-modified proteins in plasma and in cytosolic, mitochondrial and mixed membrane fractions of livers from rats treated with DF for 7 days. Further studies on the functional significance, identification of major modified proteins and the time course of these modifications are in progress.

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