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FORMATION OF TRIFLUOROACETYLATED PROTEIN ANTIGENS IN CULTURED RAT HEPATOCYTES EXPOSED TO HALOTHANE *IN VITRO*

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Abstract—Immune responses to novel, halothane metabolite-modified protein antigens (trifluoroacetylated proteins; TFA-proteins) have been implicated in the pathogenesis of halothane hepatitis. The aim of the present study was to investigate and characterize expression of TFA-proteins in cultures of rat hepatocytes which were exposed to halothane *in vitro*. Following exposure to halothane, the hepatocytes were harvested, then subcellular fractions were prepared and were analysed by immunoblotting for expression of antigens recognized by a rabbit anti-TFA antiserum, and by antibodies in sera from two patients with halothane hepatitis. Hepatocytes exposed to halothane *in vitro* were shown to express novel microsomal protein antigens, which exhibited molecular masses that were identical to the molecular masses of the major TFA-protein antigens expressed *in vivo*, in livers of halothane-treated rats (100, 80 and 60 kDa). Experiments in which hepatocytes were exposed to halothane in the presence of SKF-525A, or were exposed to deuterated halothane in place of halothane, confirmed that these novel antigens were TFA-modified proteins whose generation required cytochrome P450-mediated metabolism of halothane. The maximal levels of TFA-antigens expressed *in vitro* were about 30% of the levels expressed in halothane-treated rats *in vivo*. Maximal expression of the TFA-antigens *in vitro* occurred when hepatocytes were exposed to halothane at doses which yielded concentrations of the drug in culture medium of about 13 μM. Expression of the antigens *in vitro* occurred slowly, with an apparent half-time of about 8 hr. Overall, these results demonstrate that the properties of the TFA-antigens expressed in cultured hepatocytes *in vitro* closely resemble the properties exhibited by the antigens expressed *in vivo*, in livers of halothane-treated rats.

Key words: anaesthetics; cytochrome P450; hepatitis; reactive metabolite; immunotoxicity; drug metabolism

A very small proportion of patients anaesthetized with halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) develop severe and potentially fatal liver damage, termed "halothane hepatitis" [1]. Halothane hepatitis is believed to arise via an immune response to novel, halothane-induced liver antigens [2–4]. The novel antigens are expressed in livers of rabbits, rats and humans that have been exposed to halothane *in vivo*, and experiments performed using the technique of immunoblotting have indicated that they comprise a group of liver microsomal polypeptides which range in molecular mass from 54 to 100 kDa [5–7]. IgG† antibodies which recognise the antigens are present in sera of patients with halothane hepatitis, and are highly specific for this group of patients [4, 5].

Generation of the various antigens has been shown to involve cytochrome P450-mediated metabolism of halothane to a reactive metabolite which binds covalently to the proteins, namely TFA-chloride [6, 8, 9]. The majority of the TFA-proteins involved (100,80,63,59,58 and 57 kDa‡) have been purified and characterized biochemically [4, 10–15]. Immunoblotting experiments have shown that the patients' antibodies recognize novel epitopes on the antigens which consist of the TFA-group plus structural features of the protein carriers. In addition, these investigations indicated that the 100 and 80 kDa antigens are recognized most commonly by the patients' antibodies [4–8]. More recently, ELISA studies performed using purified antigens have shown that a high proportion of the patients' sera also have autoantibodies which recognize non TFA-modified epitopes on several of the antigens (80, 59, 58 and 57 kDa) that in general cannot be detected by immunoblotting [11, 14, 16–18].

All of the antigens characterized to date are highly abundant and long-lived peripheral membrane proteins which normally reside within hepatocytes, within the lumen of the endoplasmic reticulum [4, 19, 20]. Since cytochromes P450 are known to be located in the membrane of the endoplasmic reticulum [21], this implies that generation of the TFA-antigens occurs within hepatocytes. However,

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† Abbreviations: TFA, trifluoroacetyl; IgG, Immunoglobulin G; LDH, lactate dehydrogenase.

‡ Note that the 80 kDa antigen fraction was originally ascribed a molecular mass of 76 kDa; recently this has been corrected to 80 kDa, after purification of the antigen [Ref. 14]. In addition, the 57–63 kDa antigens co-migrate as a single, broad "60 kDa" antigen fraction when analysed using the conditions of SDS–PAGE employed in the present study.

TFA-antigens have been detected on the surface of hepatocytes isolated from livers of halothane-anaesthetized rabbits [22] and halothane-treated rats [23]. This finding is of considerable interest, since only TFA-antigens which are expressed on the cell surface should be accessible to immune effector mechanisms in patients *in vivo* [24].

In order that we might investigate in detail the subcellular trafficking of the TFA-proteins, and define the mechanisms by which they become expressed on the cell surface, recently we developed a technique for generation of TFA-antigens in cultured rat hepatocytes. Using this technique, we demonstrated that hepatocytes which had been cultured with halothane *in vitro* expressed several novel polypeptide antigens, which were recognized by a rabbit antiserum specific for the TFA-protein hapten [25]. The objectives of the present study were to determine whether the novel antigens expressed in cultured rat hepatocytes were recognized by antibodies from patients with halothane hepatitis, to investigate the subcellular location, dose dependence and kinetics of expression of the antigens, and to establish whether their expression involved cytochrome P450-mediated metabolism of halothane to TFA-chloride.

MATERIALS AND METHODS

Reagents. Halothane, deuterium oxide and sodium deuterium oxide were obtained from the Aldrich Chemical Co. (Poole, U.K.) Williams' medium E, newborn calf serum and gentamycin were purchased from Gibco BRL (Paisley, U.K.). Hydrocortisone, insulin, β-NADH, 5-bromo-4-chloro-3-indoleto-luidine phosphate and *p*-nitrobluetetrazolium were obtained from Sigma Chemical Co. (Poole, U.K.). Collagenase A (from *Clostridium histolyticum*; EC 3.4.24.3) was purchased from Boehringer Mannheim Biochemica (Lewes, U.K.). β-Diethylaminoethyl diphenylpropylacetate hydrochloride (SKF-525A) was a gift from SmithKline Beecham (Weybridge, U.K.). Human sera were obtained from two patients with halothane hepatitis and from a normal blood donor. Anti-TFA antiserum was prepared in the authors' laboratory by immunizing female New Zealand White rabbits (2.5 kg) with TFA-labelled rabbit serum albumin, according to the procedure described by Satoh *et al.* [23]. Alkaline phosphatase-conjugated antisera to human IgG and to rabbit IgG were from Tago Immunodiagnostics Ltd (CA, U.S.A.) and were obtained from Tissue Culture Services Ltd (Buckingham, U.K.).

Synthesis of deuterated halothane. Deuterated halothane was synthesized according to a modification of the procedure of Hine *et al.* [26]. Freshly distilled halothane (50 mL) was stirred in the dark with 125 mL of 40% (w/w) sodium deuterium oxide in deuterium oxide for 24 hr at room temperature. After removal of the aqueous phase, a further 125 mL of 40% (w/w) sodium deuterium oxide in deuterium oxide was added and the incubation was repeated. The aqueous phase was again discarded and the organic phase was washed three times with 20 mL of deuterium oxide, then dried over anhydrous sodium sulphate and purified by distillation. The yield of deuterated halothane was 25 mL (50%) and its purity was 98.5%, as determined by gas chromatography/mass spectrometry.

Animals and treatments. Male Sprague–Dawley rats (180–200 g) were treated with halothane by i.p. injection of a 21.5% solution in sesame oil, to give a dose of 20 mmol/kg. "Control" animals received an equivalent dose of sesame oil alone. Animals were left to recover overnight and then were killed by cervical dislocation. Livers were removed and homogenized in ice cold 15 mM Tris–HCl, pH 6.8, containing 0.25 M sucrose and 0.1 mM EDTA [27]. Microsomal fractions were prepared by differential centrifugation [27] and were snap-frozen and stored at −75°.

Isolation and culture of hepatocytes. Hepatocytes were isolated from livers of male Sprague–Dawley rats (200–250 g) using a two-step collagenase technique, as described initially by Seglen [28] and subsequently modified by Paine *et al.* [29]. Cells were plated onto 75 cm^2 culture flasks (Bibby, Stone, U.K.) at a density of 9×10^6 cells/flask, in Williams' medium E which had been supplemented with 5% newborn calf serum, 1 μM insulin, 10 μM hydrocortisone and 50 mg/L gentamycin. Cells were incubated at 37°, under a humidified atmosphere of air (95%) and CO_2 (5%). After incubation for 4 hr, the medium was replaced with the same medium containing 10 mM HEPES (pH 7.4) and flasks were closed air-tight using silicone stoppers. Halothane was injected though the stoppers onto filter paper suspended above the culture medium [25]. When small amounts of halothane were applied (<5 μL per flask), a 10% (v/v) solution of the compound in dimethyl sulphoxide was prepared and aliquots of this solution were added to the flasks. Control experiments verified that dimethyl sulphoxide did not influence the viability of the cells or their ability to express TFA-proteins. After the designated incubation period, cells were washed twice with ice-cold phosphate buffered saline, pH 7.4, and were harvested using 0.5 mL of ice-cold 0.25 mM sucrose, 15 mM Tris–acetate, pH 6.8, 0.1 mM EDTA. The harvested cells were homogenized by freeze–thawing, followed by sonication for 60 sec on ice using a probe sonicator (amplitude 8 μm) [30]. Subcellular fractions were prepared by differential centrifugation [5] and were snap-frozen by immersion in liquid nitrogen, then were stored at −75°.

Biochemical assays. Protein was determined according to Lowry *et al.* [31] with bovine serum albumin as standard. Activity of the enzyme LDH (EC 1.1.1.27) in culture media and whole cell extracts was determined according to the procedure described by Bergmeyer *et al.* [32]. The activities of NADPH-dependent cytochrome P450 reductase (EC 1.6.99.3) and succinate dehydrogenase (EC 1.3.99.1) were determined according to published methods [33, 34].

Determination of concentrations of halothane in culture media. Concentrations of halothane in the media were determined by gas–liquid chromatography, after extraction into *n*-hexane, essentially as described by Mallédant *et al.* [35]. After incubation with halothane, media were collected in glass test tubes which were tightly capped and were

immediately frozen and stored at −20° until analysis. Immediately before analysis, 0.5 mL aliquots of the media were transferred to extraction tubes containing 5 mL of *n*-hexane and the tubes were mixed for 10 min at room temperature on a Vibrax VXR Shaker, at 2000 rpm. After separation of the phases, 2 μL of the hexane phase was injected into a Pye Unicam 304 chromatograph equipped with an Alltech NON-PAKD column (15 m × 0.53 mm, 2.5 μm) (Alltech Associates Ltd, Carnforth, U.K.). The injection port temperature was set on 200°, the oven temperature was 50° and detection was by electron capture (Pye Unicam Ni^{63} detector, set to 200°). Calibration curves was constructed by dilution of halothane directly into culture media. The calibration was linear over the range 1–150 μM halothane.

SDS–PAGE and immunoblotting. Liver microsomal proteins were resolved by SDS–PAGE and were transferred electrophoretically to nitrocellulose, using procedures which have been described in detail elsewhere [5, 6, 19]. The nitrocellulose blots were developed by sequential incubation with diluted primary and secondary antisera, essentially as described previously [5, 6, 19]. However, secondary antibodies conjugated to alkaline phosphatase were used in place of secondary antibodies conjugated to horseradish peroxidase; Tween 20 (at 0.2% v/v) was included in the buffer used for the final four washes prior to colour development; and colour development was performed by incubation with 5-bromo-4-chloro-3-indoletoluidine phosphate and *p*-nitroblue-tetrazolium [36]. These modifications were introduced to increase the sensitivity of immunodetection. Gel sample loadings and antibody incubation conditions are listed in the figure legends. In order to quantify the amounts of TFA-protein antigens formed, tracks were scanned densitometrically using a Shimadzu dual wavelength TLC scanner CS-930 (V.A. Howe & Co. Ltd, Oxon, U.K.) (wavelength 600 nm, in the reflectance mode). In order to standardize the densitometric data, reference microsomal fractions obtained from livers of rats killed 18 hr after i.p. injection of halothane, at a dose of 10 mmol/kg body wt, or vehicle alone, were included on all blots.

RESULTS

Cultured rat hepatocytes were exposed to halothane by injecting the compound onto pieces of filter paper which were suspended in sealed tissue culture flasks, above the culture medium. This approach resulted in rapid, reproducible and dose-dependent partitioning of halothane into the culture medium, via the gas phase (Fig. 1). Equilibrium concentrations of halothane were reached within 15 min, and once equilibrium had been achieved the levels remained constant for up to 18 hr (Fig. 1B). The concentration of halothane achieved when media were exposed to halothane, at a dose of 0.5 μL per flask, were markedly lower in the presence than in the absence of hepatocytes (13.4 ± 1.4 μM (N = 14); and 20.7 ± 4.0 μM (N = 11), respectively) (Fig. 1B). Most probably this was because of partitioning of halothane into the hepatocytes.

Microsomal fractions prepared from hepatocytes which had been exposed to halothane *in vitro* expressed several polypeptide antigens which were not expressed in microsomal fractions from control hepatocytes. These halothane-induced antigens were recognized by antibodies in an anti-TFA rabbit antiserum and by antibodies in sera from two patients with halothane hepatitis (Fig. 2). The major halothane-induced antigens recognized by the anti-TFA antiserum had molecular masses of 100, 80 and 60 kDa (Fig. 2, panel A, *in vitro*; compare lane H with lane C), while the halothane-induced antigens recognized by the patients' antibodies had molecular masses of 100 and 80 kDa (Fig. 2, panels B and C, *in vitro*; compare lane H with lane C). The sera recognized TFA-proteins expressed in livers of rats treated with halothane *in vivo* which had identical apparent molecular masses to those of the novel antigens expressed in the cultured hepatocytes (Fig.

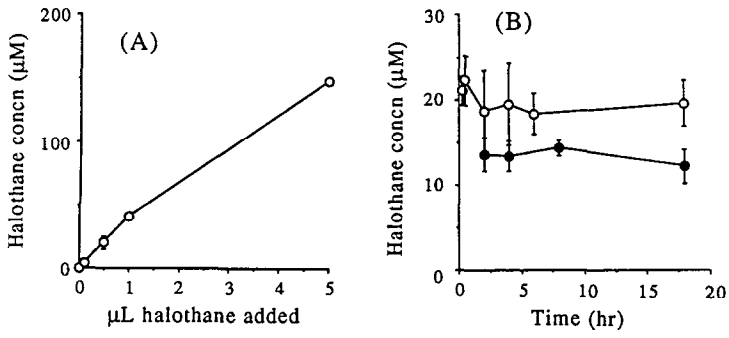

Fig. 1. Uptake of halothane into culture medium. Closed culture flasks containing 15 mL of culture medium were exposed to halothane by injecting the compound though the silicone stoppers onto filter paper suspended above the culture medium. After incubation, media were collected and were extracted with *n*-hexane and the concentrations of halothane were determined by gas chromatography. (A) Dose-dependent partitioning of halothane into culture media determined 1 hr after administration of halothane. (B) Time-dependent partitioning of halothane into culture media in the absence (open circles) or presence (solid circles) of cultured hepatocytes (9 × 10^6 cells/flask). Data are means ± SD (N = 4).

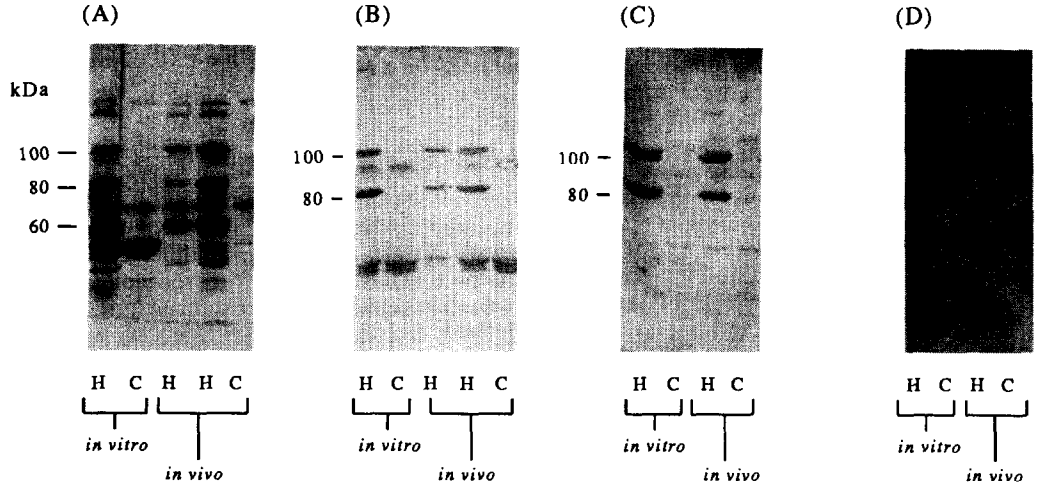

Fig. 2. Expression of halothane-induced protein antigens *in vivo* and *in vitro*. *In vivo* samples were microsomal fractions prepared from livers of rats killed 18 hr after i.p. treatment with either halothane in sesame oil (at 20 mmol/kg body wt) (H), or sesame oil alone (C). *In vitro* samples were microsomal samples prepared from hepatocytes which had been incubated for 18 hr with (H) or without (C) halothane. Sample loadings were: 40 μg protein/lane for *in vitro* fractions; 20 μg/lane and 5 μg/lane for *in vivo* (H) fractions; and 20 μg/lane for *in vivo* (C) fractions. Panels A–D are immunoblots, which were developed using anti-TFA antiserum (A), sera from two patients with halothane hepatitis (B,C) and serum from a normal blood donor (D). The molecular masses of the major halothane-induced polypeptide antigens are indicated.

2, panels A, B and C, *in vivo*; compare lane H with lane C). The various halothane-induced antigens were not recognized by antibodies in a control serum, obtained from a normal blood donor (Fig. 2, panel D).

In addition to containing antibodies to halothane-induced protein antigens, the anti-TFA antiserum and the patients' sera contained antibodies which recognized several non TFA-labelled proteins. The anti-TFA antiserum recognized a non TFA-labelled polypeptide antigen of about 50 kDa, which was expressed in microsomal fractions from hepatocytes that had not been exposed to halothane (Fig. 2, panel A, *in vitro*; lane C). In a previous investigation, we demonstrated that recognition of this 50 kDa hepatic protein antigen by the anti-TFA antiserum was due to the presence in the antiserum of a population of antibodies not directed against TFA-protein epitopes [25]. Thus, when immunoblots were developed using anti-TFA antiserum, in the presence of the hapten inhibitor *N*-ε-TFA-L-lysine (at 0.5 mM), recognition of the 100, 80 and 60 kDa TFA-proteins was abolished, but recognition of the 50 kDa antigen was unaffected [25]. Interestingly, although this 50 kDa antigen was expressed in microsomal fractions prepared from isolated rat hepatocytes, it was not detectable in microsomal fractions prepared from rat liver (Fig. 2, panel A). The anti-TFA antiserum also weakly recognized a non TFA-modified polypeptide of about 65 kDa which was expressed in all samples analysed (Fig. 2, panel A). Recognition of this antigen by the anti-TFA antibodies was also not affected by *N*-ε-TFA-L-lysine [25].

The two patients' sera contained antibodies which recognized several non TFA-modified proteins (Fig. 2, panels B and C), as did the control serum (Fig. 2, panel D). Recognition of a variety of non TFA-modified rat liver polypeptide antigens by antibodies in human sera has been observed in previous immunoblotting studies [4–7, 19, 20, 37]; the significance of these antibodies is unclear at the present time.

To investigate the subcellular locations of the halothane-induced antigens expressed in hepatocytes, subcellular fractions were prepared by differential centrifugation and were analysed by immunoblotting. Three particulate fractions, denoted nuclear, mitochondrial and microsomal, and a soluble (cytosolic) fraction were obtained. Enzyme analyses confirmed that NADPH-dependent cytochrome P450-reductase, which is known to be situated in the endoplasmic reticulum [21], was concentrated in the microsomal fraction and that the highest activities of the mitochondrial enzyme marker succinate dehydrogenase [21] were present in the mitochondrial fraction (Table 1). The immunoblotting experiments revealed that the various TFA-antigens were concentrated in the microsomal fraction, with lesser amounts detectable in the mitochondrial fraction, and very low levels evident in the nuclear fraction (Fig. 3). This pattern of antigen distribution closely paralleled the pattern of distribution of NADPH-dependent cytochrome P450 reductase activity (Table 1).

Expression of the halothane-induced antigens was markedly dependent upon the concentration of halothane in the culture medium. Maximal levels of antigen expression were observed following addition of halothane at a dose of 0.5 μL per flask (Fig. 4;

Table 1. Activities of enzyme markers in subcellular fractions prepared from cultured hepatocytes

Fraction	NADPH-dependent cytochrome P450 reductase (nmol/min/mg protein)		Succinate dehydrogenase (nmol/min/mg protein)	
	Control	Halothane	Control	Halothane
Nuclear	6.5 ± 2.5	5.7 ± 1.4	18.0 ± 3.2	24.7 ± 1.9
Mitochondrial	15.9 ± 1.2	13.9 ± 5.7	33.1 ± 3.2	45.7 ± 5.1
Microsomal	49.8 ± 3.9	54.7 ± 3.1	21.4 ± 2.5	26.0 ± 2.6
Cytosolic	8.2 ± 0.7	6.5 ± 1.4	14.2 ± 2.9	12.1 ± 0.7

Subcellular fractions were obtained from hepatocytes incubated for 18 hr either with (halothane) or without (control) halothane, at 0.5 μL/flask. Values are means ± SD (N = 3).

note that very similar results were obtained in a further three independent experiments). As mentioned previously, this yielded a concentration of halothane in the culture medium of 13.4 μM, at 1 hr after addition of halothane. Addition of higher doses of halothane (up to 5 μL per flask, which yielded a concentration of halothane in the culture medium of 170 μM) did not result in increased levels of antigen expression. None of the doses of halothane used in these studies had any detectable influence upon cell viability, which was greater than 95% in all cases as assessed from leakage of lactate dehydrogenase into the culture media.

Following injection of 0.5 μL of halothane per flask, expression of the halothane-induced antigens occurred relatively slowly. Thus, only very low levels of the antigens were detectable after 4 hr, and thereafter levels of antigen expression increased progressively up to 16 hr (Fig. 5; very similar results were obtained in a further two independent experiments). The levels of halothane-induced antigens expressed in microsomal fractions prepared from hepatocytes exposed to halothane for 18 hr, at a dose of 0.5 μL per flask, were highly reproducible and were 31 ± 6% of the levels of the antigens expressed in microsomal fractions from livers of rats sacrificed 18 hr after exposure to halothane *in vivo*, at a dose of 20 mmol/kg, as determined by densitometric scanning of immunoblots (mean ± SD of results obtained in five independent hepatocyte experiments).

To verify that expression of the various halothane-

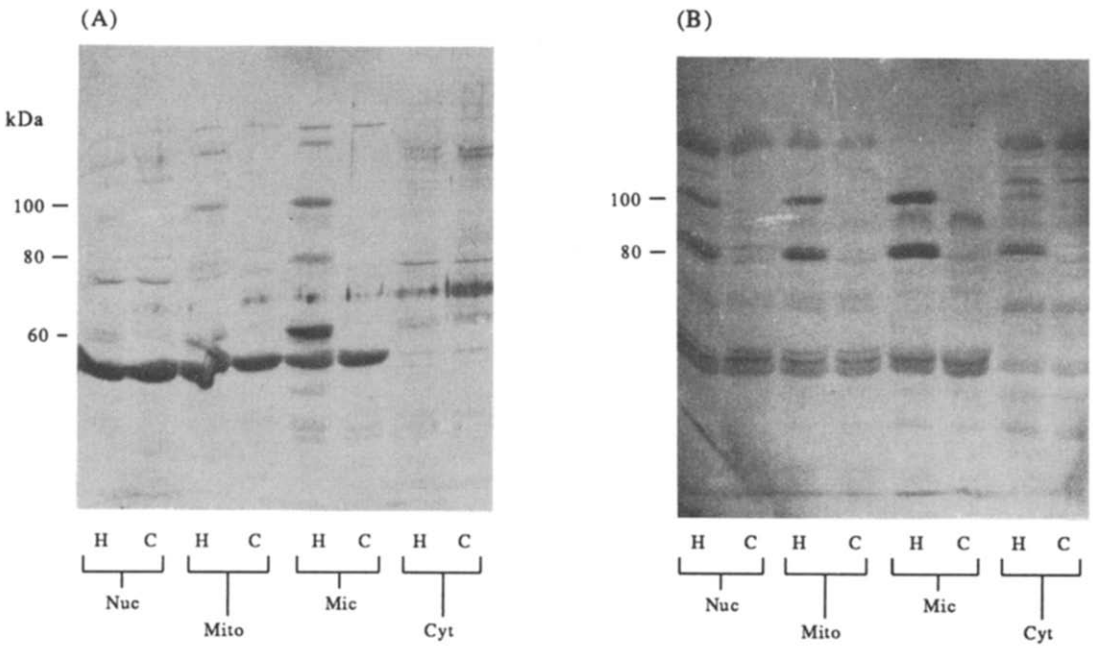

Fig. 3. The subcellular distribution of the halothane-induced antigens expressed in hepatocytes. Subcellular fractions (Nuc, nuclear; Mito, mitochondrial; Mic, microsomal; Cyt, cytosolic) prepared from hepatocytes which had been incubated with (H) or without (C) halothane (0.5 μL/flask) for 18 hr were analysed by immunoblotting. Sample loading was 40 μg protein/lane. Immunoblots were developed with anti-TFA antiserum (A) or serum from a patient with halothane hepatitis (B).

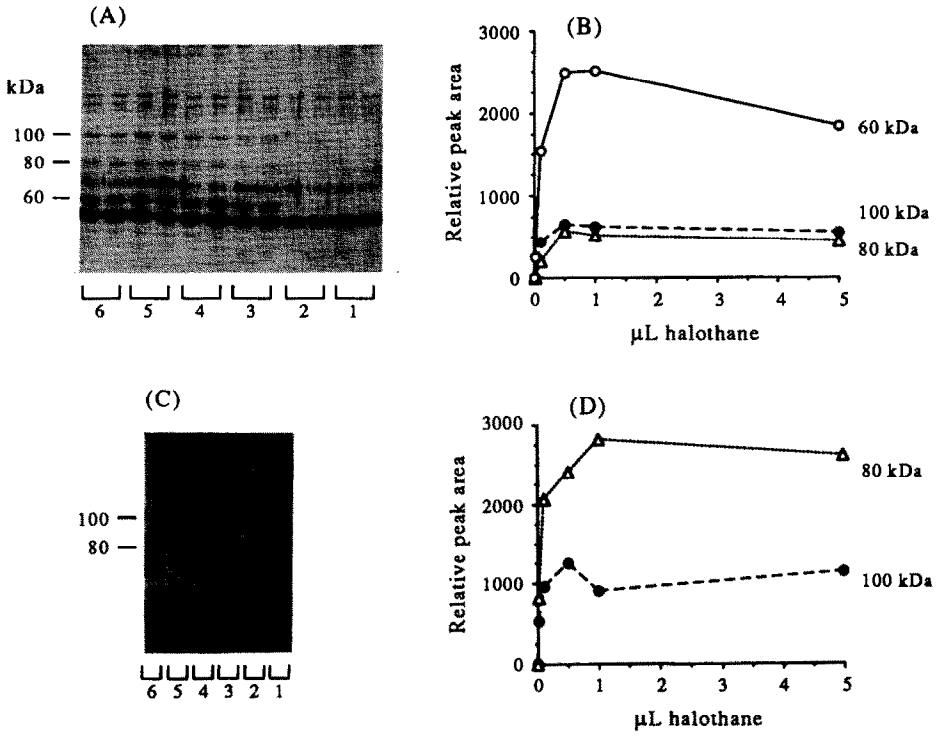

Fig. 4. The influence of concentration of halothane on expression of halothane-induced antigens in hepatocytes. Microsomal fractions prepared from hepatocytes incubated for 18 hr with various concentrations of halothane were analysed by immunoblotting. Sample loading was 40 μg protein/lane. (A,C) Immunoblots, which were developed using anti-TFA antiserum (A) or patient's serum (C). The doses of halothane added per flask were: 0, 0.01, 0.1, 0.5, 1.0, and 5.0 μL, for lanes 1–6 respectively. (B,D) Densitometric scans of the halothane-induced antigen bands evident in blots A and C.

induced polypeptide antigens involved oxidative, cytochrome P450-mediated metabolism of halothane, two further experiments were performed. Firstly, hepatocytes were exposed to halothane in the presence of an inhibitor of cytochrome P450, namely SKF-525A: this abolished antigen expression (Fig. 6). This result could not be attributed to cellular cytotoxicity, because cell viability was greater than 90% in all groups of cells analysed, regardless of exposure to halothane and/or SKF-525A. Secondly, antigen expression in hepatocytes exposed to deuterated halothane, in place of halothane, was investigated. Previously, deuteration of halothane has been shown to reduce its rate of oxidative, cytochrome P450-mediated metabolism [38]. Expression of the halothane-induced antigens was indeed reduced markedly when cells were exposed to deuterated halothane (Fig. 7). Densitometric scanning revealed that the magnitude of the deuterium isotope effect was greater when nitrocellulose blots were developed using anti-TFA antiserum (40–45%; Fig. 7, panel A) than when blots were developed using a patient's serum (about 35%; Fig. 7, panel B); this same pattern of results was obtained in three independent experiments.

DISCUSSION

We have reported previously [25] that cultured rat hepatocytes which had been exposed to halothane *in vitro* express novel, halothane-induced polypeptide antigens which are recognized by a rabbit antiserum specific for a covalently bound metabolite hapten derived from halothane (the TFA-group). These polypeptide antigens exhibited the same apparent molecular masses as the major TFA-protein antigens expressed in livers of rats treated with halothane *in vivo* (100, 80 and 60 kDa) [25]. In the present study, we have shown that the 100 and 80 kDa halothane-induced antigens expressed *in vitro* are recognized by antibodies in sera from patients with halothane hepatitis, but not by antibodies in a control human serum sample. Furthermore, we found that expression of the various antigens was abolished when hepatocytes were exposed to halothane in the presence of an inhibitor of cytochromes P450, namely SKF-525A, and that antigen expression was reduced markedly when hepatocytes were exposed to deuterated halothane in place of halothane. Substitution of the single hydrogen atom of halothane by deuterium has been shown to reduce the rate of oxidative, cytochrome P450-mediated metabolism of the drug to TFA-chloride [6, 23, 38]. In view of these various findings, we conclude that the novel antigens expressed when hepatocytes are exposed to halothane *in vitro* are TFA-modified proteins, which are indistinguishable immunochemically from the TFA-protein antigens expressed *in vivo* in livers of halothane-exposed animals and humans [2–7].

The magnitude of the deuterium isotope effect

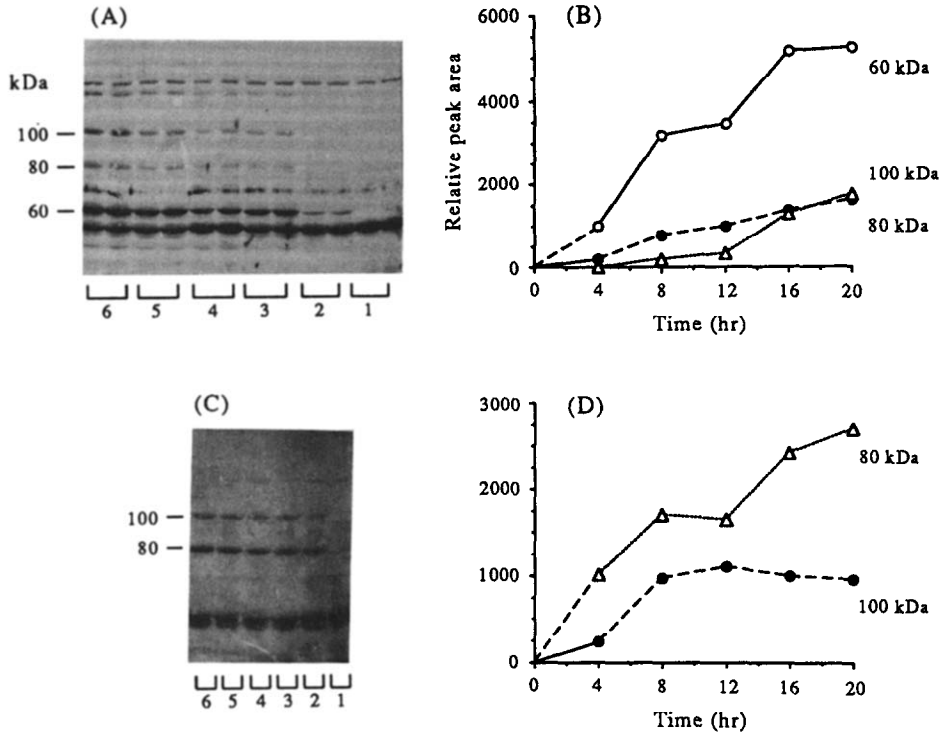

Fig. 5. The kinetics of expression of halothane-induced antigens in hepatocytes. Microsomal fractions prepared from hepatocytes which had been incubated with halothane (0.5 μL/flask) for between 4 and 20 hr, or without halothane for 20 hr, were analysed by immunoblotting. (A,C) Immunoblots, which were developed using anti-TFA antiserum (A) or patient's serum (C). (B,D) Densitometric scans of blots A and C. Sample loading was 40 μg protein/lane. Samples were: hepatocytes incubated without halothane for 20 hr (lanes 1); and hepatocytes incubated with halothane for 4, 8, 12, 16 and 20 hr (lanes 2–6, respectively).

observed in the present studies was no more than 35–45%. This is markedly less than the deuterium isotope effect observed in a previous study, when expression of TFA-protein antigens was investigated in livers of rats which had been exposed to halothane and deuterated halothane *in vivo* and in rat liver microsomal fractions which had been incubated with the compounds *in vitro* [6]. Deuterium isotope effects are kinetic in nature, and so the magnitude of the effect decreases as the incubation time is increased. Previously, an incubation time of 2 hr was used for the *in vitro* microsomal incubations, while rats were sacrificed 4 hr after treatment with halothane or deuterated halothane for the *in vivo* experiments [6]. In contrast, in the present study hepatocytes were exposed to the compounds for 8 hr. This period of time was necessary to ensure expression of detectable levels of the TFA-antigens.

Moreover the previous *in vivo* experiments were undertaken using rats which had been pre-treated with phenobarbitone, to induce metabolism of halothane [6]. Subsequent to this, pretreatment of rats with phenobarbitone was shown to change the pattern of TFA-protein antigens expressed following exposure of the animals to halothane, and to decrease levels of expression of the TFA-antigens recognized by antibodies from patients with halothane hepatitis [19]. Presumably this is because, in phenobarbitone-pretreated rats, halothane is metabolized by induced cytochrome P450 isozymes which are different from those responsible for metabolism of halothane in untreated rats. A likely consequence of this might be a markedly different deuterated isotope effect in phenobarbitone-treated rats, as compared with untreated rats. Recently we have analysed liver microsomal fractions from groups of uninduced rats, which were killed 3 hr after i.p. treatment with halothane or deuterated halothane, for expression of TFA-proteins. In such animals, the magnitude of the deuterium isotope effect was 40–50% (T.L. Knight, F.N.A.M. van Pelt and J.G. Kenna, unpublished results).

Neither of the two patients' sera used in the present study recognized TFA-protein antigens of 60 kDa. In contrast, the anti-TFA rabbit antiserum bound strongly to TFA-proteins exhibiting this molecular mass. This apparent discrepancy has been observed in previous studies, and is a consequence of the different epitope specificities of the respective antibodies [2–4, 6, 19]. Rabbit anti-TFA antisera are raised by immunizing rabbits with TFA-labelled rabbit serum albumin and recognize the TFA hapten bound covalently to any protein carrier [6, 23]. In contrast, the patients' antibodies recognize novel epitopes consisting of the TFA hapten plus specific structural features of the individual protein carriers

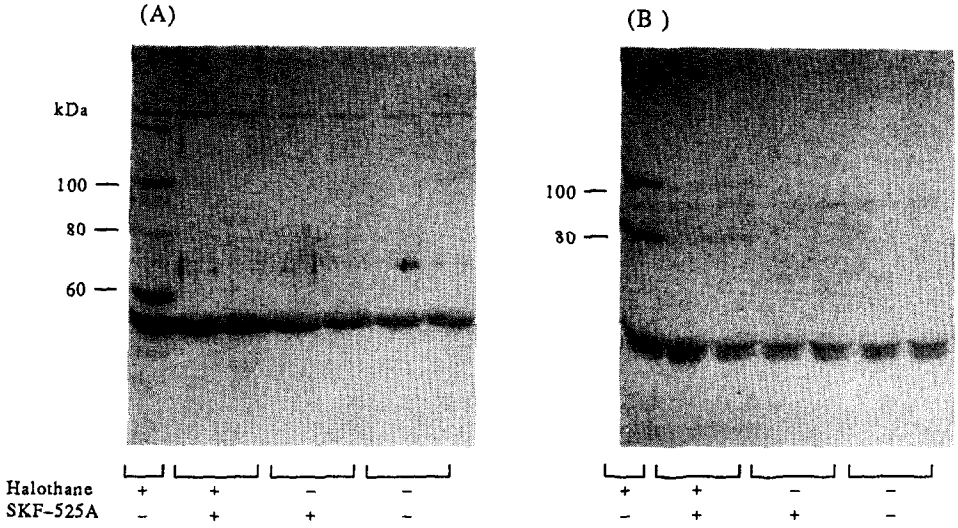

Fig. 6. Inhibition of expression of halothane-induced antigens in hepatocytes by SKF-525A. Microsomal fractions prepared from hepatocytes which were incubated for 30 min with (+) or without (−) 10 μM SKF-525A, then for a further 8 hr in the absence (−) or presence (+) of halothane, were analysed by immunoblotting. Sample loading was 40 μg protein/lane. Immunoblots were developed using anti-TFA antiserum (A) or a patient's serum (B).

[6]. As detected by immunoblotting, antibodies to TFA-proteins of 80 and 100 kDa are commonly present in patients' sera, but antibodies to TFA-proteins of 60 kDa are relatively rare [4–6]. The difference between the epitope specificities of the antibodies may explain why the deuterium isotope effect observed with anti-TFA antiserum was greater than that observed with the patients' sera (Fig. 7).

Recently Gut and co-workers have reported that antibodies specific for the TFA-hapten recognize two constitutive, non TFA-modified proteins, which have molecular masses of 64 and 52 kDa and are

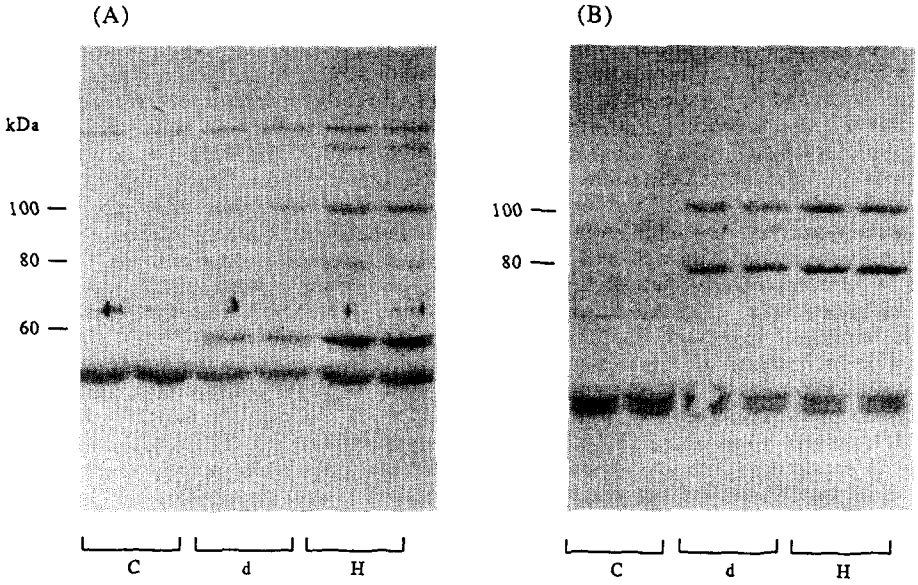

Fig. 7. Expression of halothane-induced antigens in hepatocytes exposed to deuterated halothane vs halothane. Microsomal fractions prepared from hepatocytes incubated for 8 hr with halothane (H) or deuterated halothane (d) (both at 0.5 μL/flask), or without halothane (C), were analysed by immunoblotting. Sample loading was 40 μg protein/lane. Immunoblots were developed with anti-TFA antiserum (A) or a patient's serum (B).

expressed in various tissues from rats and humans not previously exposed to halothane [38–40]. Recognition of the constitutive proteins by anti-TFA antibodies was inhibited in the presence of *N*-ε-TFA-L-lysine at 1 mM and one of the proteins was shown to be the E2 subunit of the pyruvate dehydrogenase complex [39–41]. This protein contains a lipoyl-lysine prosthetic group, and its recognition by anti-TFA antibodies was shown to be due to immunochemical cross-reactivity between TFA-lysine and lipoyl-lysine [40, 41]. In view of this, it is interesting to note that the anti-TFA antiserum used in the present study recognized polypeptide antigens of about 50 and 65 kDa which were expressed in subcellular fractions from hepatocytes not exposed to halothane (Fig. 1, panel A). However, we found previously that recognition of these two polypeptides by our anti-TFA antiserum was not inhibited in the presence of *N*-ε-TFA-L-lysine [25]. We conclude that their recognition is attributable to the presence in our antiserum of antibodies not directed against TFA-protein epitopes, and that these polypeptides are quite distinct from the cross-reactive proteins described by Gut *et al.* [39–41].

Our investigations of the subcellular location of the TFA-antigens produced in cultured hepatocytes has shown that they are concentrated in the microsomal fraction, as prepared by differential centrifugation, with lesser amounts present in other subcellular fractions. Analysis of the activity of NADPH-dependent cytochrome P450 reductase in the subcellular fractions revealed that the low levels of the antigens detectable in other fractions was attributable to microsomal contamination of the fractions. A very similar pattern of results was obtained in previous studies, in which the distribution of halothane-induced antigens in subcellular fractions prepared from livers of halothane-anaesthetized rabbits, and characterized by marker enzyme analysis, was investigated [5]. Since the microsomal fraction consists mainly of membrane vesicles derived from the endoplasmic reticulum [21], this suggests that the antigens are concentrated in the endoplasmic reticulum. Concentration of the antigens in the endoplasmic reticulum is consistent with the known subcellular locations of the various protein carriers and of cytochromes P450 [2–4, 19, 20]. Clearly the exact subcellular locations of the antigens, their subcellular trafficking and their mechanisms of expression on the hepatocyte surface membrane are crucial issues which will need to be investigated further in the future, using immunocytochemical techniques. In particular, it will be important to determine whether the antigens became translocated from the endoplasmic reticulum to the hepatocyte surface by membrane flow, or whether their expression on the cell surface might be a consequence of cell surface expression of cytochromes P450 [42].

Our results have also shown that expression of the TFA-antigens in hepatocytes *in vitro* occurred relatively slowly, with an apparent half-time of about 8 hr. Previously we studied the rate of degradation of TFA-antigens expressed in cultured hepatocytes *in vitro*, and showed that this was a slow process which occurred with a half-time of days [25]. These findings regarding the kinetics of antigen expression *in vitro* are extremely consistent with results obtained when the kinetics of antigen expression was investigated in halothane-treated rats *in vivo* [19]. Only very low levels of the major TFA-antigens were detected in rat liver at 4 hr after halothane treatment *in vivo*, when compared with levels of the antigens expressed after 18 hr, and subsequently the antigens disappeared relatively slowly and were still detectable after 7 days. The long lifetimes of the antigens apparently reflect the slow rates of turnover of the respective protein carriers [4]. Presumably the quite slow rate of antigen expression is due to a slow rate of TFA-labelling of the proteins, which continues progressively over a period of many hours.

Expression of the TFA-antigens *in vitro* was found to be concentration-dependent and saturable, with maximal antigen expression observed at concentrations of halothane in the culture medium of about 13 μM. This confirms and extends our previous observation that, when cultured rat hepatocytes were exposed for 18 hr to halothane at a concentration of 0.56 mM, expression of TFA-proteins was markedly lower than when hepatocytes were exposed to a concentration of 0.17 mM [25]. In neither of our studies could the concentration-dependence of antigen formation be attributed to the cytotoxicity of halothane, or its metabolites, and other workers have shown that the cytotoxic threshold of halothane in cultured hepatocytes is approximately 1 mM [35].

The reduced antigen expression seen at the higher doses of halothane might be related to depression of protein synthesis, which has been reported in isolated hepatocytes exposed to 0.1 mM halothane for 24 hr [35]. However, in view of the long lifetimes of the TFA-labelled antigens and of the unlabelled carrier proteins [4, 19], it is unlikely that modest depression of protein synthesis would effect the levels of the proteins markedly within 18 hr. More probably, oxidative metabolism of halothane by the cytochrome P450 isozymes responsible for generation of the TFA-proteins (apparently P4502E1 [19]) becomes saturated at about 13 μM. Concentration-dependent inhibition of metabolism of halothane *in vivo* has been reported in rats, guinea pigs and humans [43–45]. In addition, covalent binding of halothane metabolites to hepatic proteins and lipids was found to be markedly greater when guinea pigs were exposed inhalationally to 0.1% halothane than when animals were anaesthetized with 1% halothane [44]. Moreover investigations of expression of TFA-protein antigens in livers of rats treated i.p. with halothane *in vivo*, at doses of 1, 10, 20 and 40 mmol/kg body weight, have shown that expression of TFA-antigens is dose-dependent and maximal at doses of 20 mmol/kg (J.G. Kenna, J.L. Martin and L.R. Pohl, unpublished results).

Overall, our results have established that the properties of the TFA-protein antigens expressed in cultured rat hepatocytes *in vitro* are not noticeably different to the properties of the antigens expressed *in vivo*, in livers of halothane-exposed rats. In view of this, we feel that isolated hepatocytes are suitable for more detailed investigation of the mechanisms underlying the formation and processing of the TFA-antigens, and of their subcellular trafficking.

Particular advantages of isolated hepatocytes are that they can be exposed to the drug under rigorously controlled conditions, which can be manipulated much more readily than in *in vivo* experiments, and that isolated cells can be used directly in immunohistochemical studies. Furthermore, this finding raises the intriguing possibility that cultured hepatocytes might be of value for identification and characterization of metabolite-induced antigens associated with drug toxicities other than halothane hepatitis [46–50]. If so, this would be a great advantage, since simple and well-standardized techniques for characterization of such antigens have yet to be described.

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