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HUMAN HEPATOCYTES EXPRESS TRIFLUOROACETYLATED NEOANTIGENS AFTER *IN VITRO* EXPOSURE TO HALOTHANE

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Abstract—Biotransformation of anaesthetic halothane by cytochrome P450-dependent monooxygenases resulted in the production of reactive intermediate trifluoroacetyl (TFA) halide, capable of covalently binding to hepatocyte proteins. TFA-modified liver proteins can act as antigens and are implicated in the pathogenesis of halothane hepatitis in humans. The aim of this study was to investigate the formation of TFA-neoantigens in halothane-treated primary cultures of adult human hepatocytes and to evaluate the usefulness of this *in vitro* model for studying immune-mediated halothane hepatotoxicity. Cultured human hepatocytes were incubated with halothane under constant temperature, atmosphere and anaesthetic concentration conditions. The results obtained show that halothane-treated hepatocytes isolated from seven different donors produced TFA-antigens as detected by immunocytochemical and western immunoblot analysis using rabbit anti-TFA antiserum. TFA-adducts were localized mainly in the endoplasmic reticulum and in small amounts on the plasma membrane of parenchymal cells. By immunoblotting, several neoantigens, with molecular masses from 42 to 100 kDa, were detected in halothane-exposed hepatocytes. These observations are consistent with the formation of TFA-adducts through metabolism of the anaesthetic and suggest that primary cultures of human hepatocytes represent a suitable *in vitro* model to study the pathogenesis of immune-mediated halothane hepatotoxicity.

Key words: human hepatocytes; primary culture; halothane metabolism; trifluoroacetylated antigens; expression; electron microscopy

The anaesthetic halothane (CF3-CHClBr) is known to cause a severe form of hepatotoxicity, commonly termed HH‡ in a very small fraction of exposed patients [1]. HH is recognized as an immunological disease similar in its fundamental aspects to other drug hypersensitivity reactions and is due to an immune response directed against halothane-altered liver antigens [2–4]. At least five polypeptides reacting with sera from HH patients have been demonstrated by immunoblotting analysis. These polypeptides are predominantly expressed in the microsomal fraction of the liver. Halothane-induced neoantigens can also react with a hapten-specific anti-TFA antibody [5–7]. The formation of these neoantigens is associated with oxidative metabolism of halothane by the cytochrome P450 system and involves the generation of highly reactive intermediate TFA groups capable of covalently binding to liver proteins [8–10]. Most studies on the mechanism of immune-mediated halothane hepatotoxicity have been performed *in vivo* using rat, rabbit and guinea pig models, which are far from representing relevant models of this anaesthetic-induced hepatitis in humans [11, 12]. Only a few observations report the generation of halothane-induced neoantigens in human liver [13]. In addition, different processes involved in the mechanism of halothane hepatotoxicity are difficult to be separately investigated *in vivo*. Therefore, it was decided to evaluate whether cultured human hepatocytes could represent a suitable model to study the different steps of immune-mediated halothane hepatotoxicity in well-defined experimental conditions.

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‡ Abbreviations: HH, halothane hepatitis; TFA, trifluoroacetyl; RSA, rabbit serum albumin; IgG, Immunoglobulin G; FCS, foetal calf serum.

MATERIALS AND METHODS

Preparation of anti-TFA–RSA antibodies. The trifluoroacetylation of RSA was conducted according to the general procedure of Goldberger and Anfinsen [14] as modified by Satoh *et al.* [15]. Anti-TFA–RSA serum was prepared by immunizing female New Zealand white rabbits with TFA–RSA. Antibody titers and specificity of anti-TFA–RSA serum were determined by ELISA. The microtiter plates were coated with 100 μL of TFA–RSA or RSA alone (10 μg/mL in 0.05 M carbonate buffer, pH 9.6) and incubated overnight at 4°. PBS containing 0.5% casein was added to block unbound sites on the surface of the microtiter wells. Different dilutions of anti-TFA–RSA serum in PBS–Tween 20 (0.05%, v/v) were added to plates. After 2 hr of incubation at room temperature plates were washed with PBS–Tween. Goat anti-rabbit IgG-peroxidase conjugate (Sigma Chemical Co., St Louis, MO,

U.S.A.), diluted 1:25,000 in PBS–Tween, was added to the plates. After 45 min of incubation at room temperature the plates were washed with PBS–Tween. The peroxidase reaction solution (100 μL of a mixture of 0.4 mg/mL *o*-phenylenediamine and 0.015% H_2O_2 in citrate–phosphate buffer, pH 5.0) was added to the plates and incubation lasted 10–15 min at room temperature. The reaction was stopped by addition of 50 μL of 2.5 M H_2SO_4 and O.D. was determined at 492 nm. The specificity of anti-TFA–RSA serum against the TFA group was confirmed by *N*-ε-TFA-L-lysine (Bachem, Bubendorf, Switzerland) inhibition in ELISA for TFA–RSA. Diluted anti-TFA–RSA serum was incubated with increasing concentrations of *N*-ε-TFA-L-lysine or *N*-ε-acetyl-L-lysine for 18 hr at 4° and then tested towards TFA–RSA in ELISA.

Isolation of human hepatocytes and in vitro *exposure to halothane.* Human hepatocytes were obtained from seven adult human donors not previously exposed to halogenated inhalation anaesthetics. They were isolated by the two-step collagenase perfusion method [16]. Cells were seeded either in 35 mm Petri dishes for immunocytochemical analysis or in 25 cm^2 flasks for western immunoblot analysis in 75% minimal essential medium and 25% medium 199 supplemented with 0.1 mg/mL BSA, 10 μg/mL bovine insulin and 10% FCS. After 18–24 hr the medium was changed to serum-free medium, containing 70 μM hydrocortisone hemisuccinate. Cultures were placed in incubator chambers at 37° under an atmosphere of 20% O_2, 75% N_2 and 5% CO_2. Halothane (Laboratoires Belamont, Paris, France) flowed through 1–2 vaporized chambers (Fluotec 3, Ohmeda) placed at the entrance to the chamber, and a carefully calibrated halogen monitor (Servo gas monitor 120, Siemens) determined the concentration of the anaesthetic. The chambers were sealed when the desired concentration was obtained. Hepatocytes were incubated under an atmosphere of halothane for 4–48 hr. Control cultures were maintained under the same conditions but without halothane.

Indirect immunolocalization of TFA adducts. Cultured hepatocytes in 35 mm Petri dishes were washed with PBS and then fixed in 4% paraformaldehyde buffered with a 0.1 M sodium cacodylate, pH 7.4 for 30 min at 4°. After a 1 hr incubation in 10% FCS in PBS to reduce non-specific binding the samples were incubated with rabbit anti-TFA–RSA serum diluted 1:400 for 1.5 hr at room temperature. Normal rabbit serum was used as control. All sera were previously absorbed against freshly isolated normal human hepatocytes. After three washes (10 min each) the samples were incubated with peroxidase-conjugated anti-rabbit IgG. Following three washes with PBS, staining was performed with 3,3′-diaminobenzidine/H_2O_2 for 20 min. To increase antibody penetration saponin was used at 0.1% concentration and added to all solutions. In some experiments bound rabbit anti-TFA–RSA antibodies were detected using biotinylated goat anti-rabbit IgG antibodies followed by streptovidin–biotinylated peroxidase complex (Amersham, U.K.). For electron microscopy analysis samples were postfixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 5 min followed by incubation in a 0.3 M glycine solution for 10 min before peroxidase staining. After staining, cells were fixed in a 1% osmium tetroxide solution in cacodylate buffer for 30 min, dehydrated in graded ethanols and embedded in Epon.

Gel electrophoresis and western immunoblot analysis. After washing with PBS hepatocytes were collected in PBS containing 0.5 mM PMSF by scraping. The cells were centrifuged and the pellet was then resuspended and sonicated in an ice-cold homogenization buffer (20 mM Tris–HCl, pH 6.8, 137 mM NaCl, 1 mM EDTA, 0.5 mM PMSF and 20 μg/mL aprotinin) and aliquots were stored at −80°. Total protein concentration was determined by the Bio-Rad protein assay, using BSA as a standard. Hepatocyte homogenates were diluted with reducing sample buffer (125 mM Tris–HCl, pH 6.8, containing 4% SDS, 10% β-mercaptoethanol, 0.04% Bromophenol blue and 20% glycerol) and heated to 95° for 10 min. The samples were separated according to Laemmli [17] through 4% stacking gel and 10% resolving gel (10 cm long and 1.5 mm thick) at 20 mA per gel. Protein loading was 100 μg/lane. The separated proteins were transferred electrophoretically to nitrocellulose membrane (Hybond-C, Amersham, U.K.) at 2.5 mA/cm^2 using MilliBlot-SDE unit (Millipore Corporation, Bedford, MA, U.S.A.) according to the manufacturer's recommendations. Nitrocellulose was blocked for 3 hr with TBS–Tween 20 (0.1%, v/v) containing 5% dry milk powder followed by incubation with anti-TFA–RSA diluted 1:2000 in TBS–Tween containing 0.5% casein for 1.5 hr at room temperature. After four washes for 10 min with TBS–Tween (0.5%, v/v) nitrocellulose was incubated with goat anti-rabbit IgG-peroxidase conjugate diluted 1:10,000 in casein buffer for 1 hr at room temperature. Detection of peroxidase activity was performed with the enhanced chemiluminescence western blotting detection system (Amersham, U.K.)

RESULTS

Specific binding to TFA group of the rabbit anti-TFA–RSA serum was demonstrated by inhibition of antiserum binding to microtiter plates coated with TFA–RSA in the presence of *N*-ε-TFA-L-lysine using an ELISA technique. By contrast, *N*-ε-acetyl-L-lysine was much less effective as inhibitor (Fig. 1). Isolated human hepatocytes were cultured for 24–48 hr under an atmosphere of 20% O_2, 75% N_2, 5% CO_2 and 1.5% halothane. After equilibrium had been achieved the concentration of halothane in the culture medium was 0.2 mM, as determined by GLC. This concentration was not toxic to human hepatocytes according to morphological criteria, activity of intra- and extracellular lactate dehydrogenase and protein synthesis and secretion rates [18]. About 70–90% of hepatocytes incubated with halothane showed cytoplasmic staining of varying intensity with anti-TFA–RSA serum (Fig. 2A and B). Immunoreactivity of halothane-treated hepatocytes was abolished when anti-TFA–RSA serum was replaced by normal rabbit serum or anti-

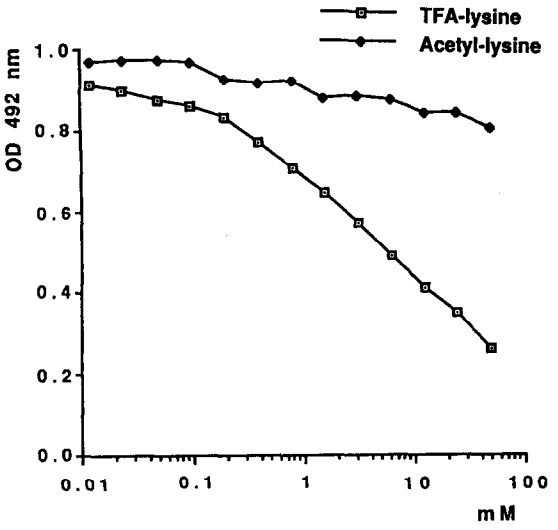

Fig. 1. Characterization of anti-TFA–RSA serum specificity. Anti-TFA–RSA was diluted 1:20,000 and incubated for 18 hr at 4° with increasing concentrations of *N*-ε-TFA-L-lysine or *N*-ε-acetyl-lysine. The mixtures were tested in ELISA for binding to TFA–RSA. The results represent O.D. readings at 492 nm.

TFA–RSA serum preincubated with *N*-ε-TFA-L-lysine. Either no immunoreactivity (Fig. 2C) or, in some cases, weak immunoreactivity was observed in hepatocytes maintained in the absence of halothane and stained with anti-TFA–RSA serum. Electron microscopy examination revealed the presence of electron-dense deposits located mainly in the endoplasmic reticulum (Fig. 3A). Less intense labelling was observed on the plasma membranes of halothane-exposed hepatocytes (Fig. 3B). In all cases mitochondria and nuclei were constantly negative. No labelling was evidenced in non-exposed cells with anti-TFA–RSA serum (Fig. 3C). Similar patterns of staining were observed in hepatocyte cultures from seven donors after exposure to halothane.

Generation of TFA-neoantigens in human hepatocytes exposed to halothane was confirmed by immunoblotting with anti-TFA–RSA serum. Halothane-treated hepatocytes expressed several neoantigens with apparent molecular weights of 100, 78, 76, 60/59, 57 and 42 kDa that were not detected prior to halothane treatment or in cells cultured in the absence of halothane (Fig. 4). The most abundant TFA-labelled polypeptide(s) formed the band at 60/59 kDa. In addition, anti-TFA–RSA serum recognized an antigen of 54 kDa in both halothane-exposed and control hepatocytes. Neoantigen formation was demonstrated in human hepatocytes treated with halothane for 4 hr and the amount was increased after a 24 hr incubation.

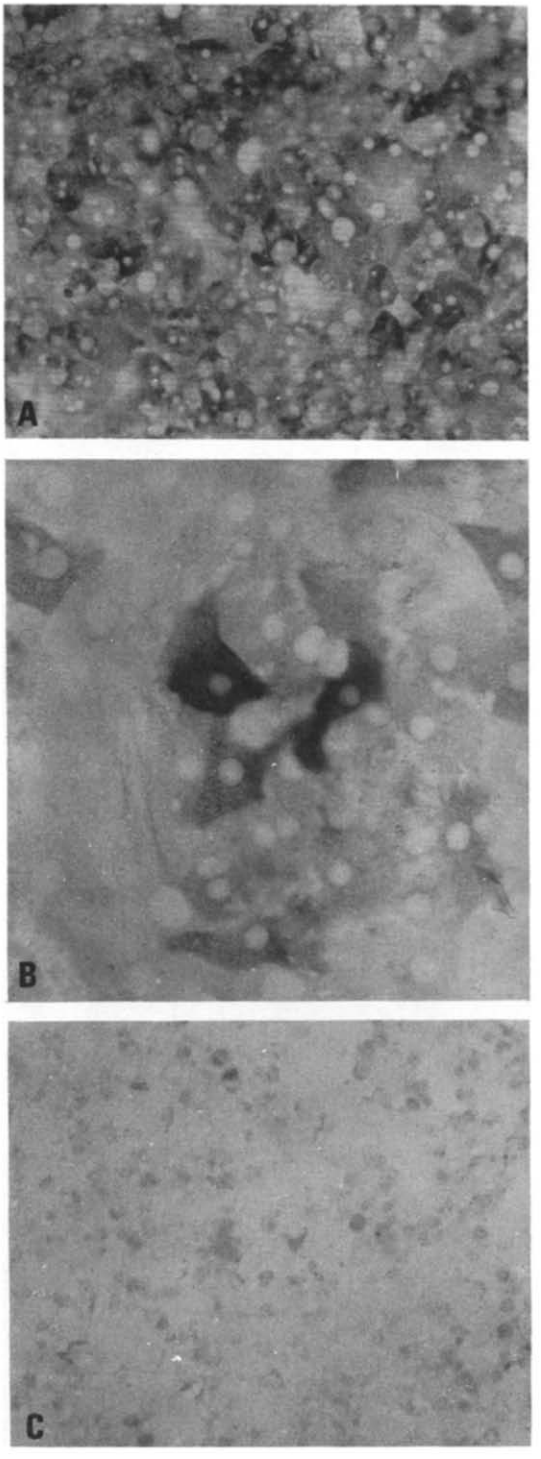

Fig. 2. Indirect immunolocalization of TFA-antigens in halothane-exposed human hepatocytes. Cultured human hepatocytes were incubated for 24 hr under an atmosphere containing 1.5% halothane (A and B). Control cultures were maintained under the same conditions but without the anaesthetic (C). After fixation cells were stained with anti-TFA–RSA serum followed by peroxidase-conjugated anti-rabbit IgG. A strong positive reaction was observed in a fraction of hepatocytes (A and B), no staining was visible in untreated hepatocytes (C). A and C: ×145; B: ×290.

DISCUSSION

Recent investigations suggest that the pathogenesis of HH involves an immune response against liver

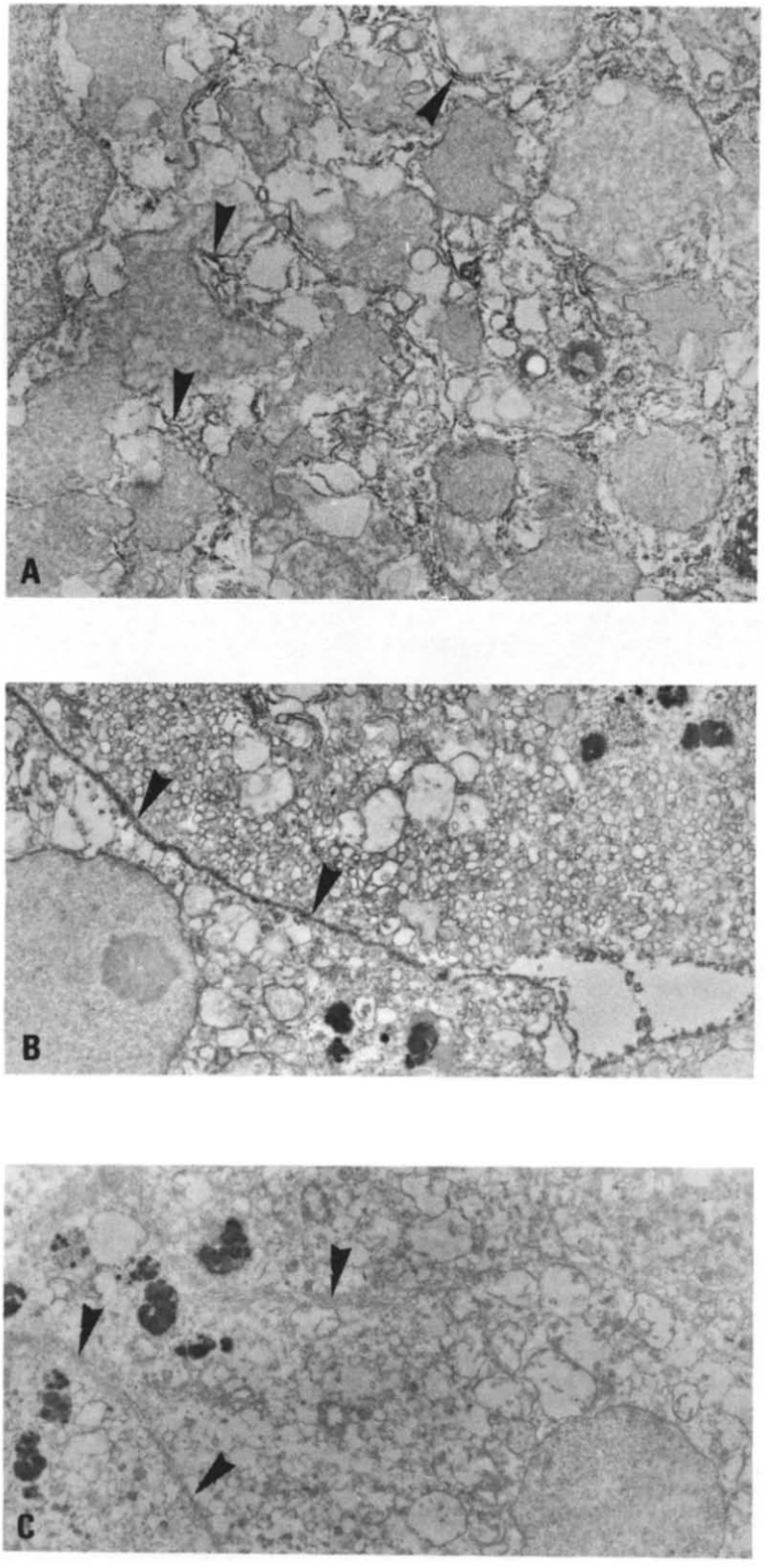

Fig. 3. Electron microscopy localization of TFA-adducts in halothane-exposed human hepatocytes. Cultured human hepatocytes were incubated for 24 hr under an atmosphere containing 1.5% halothane (A and B). Control cultures were maintained under the same conditions but without the anaesthetic (C). After fixation cells were stained with anti-TFA–RSA serum followed by biotinylated goat anti-rabbit IgG and streptovidin–biotinylated peroxidase complex. Arrows point to electron-dense deposits located in the endoplasmic reticulum (A) and on the plasma membrane (B) of halothane-treated hepatocytes. No labelling was observed in non-exposed hepatocytes (C). A: ×10,000; B and C: ×5000.

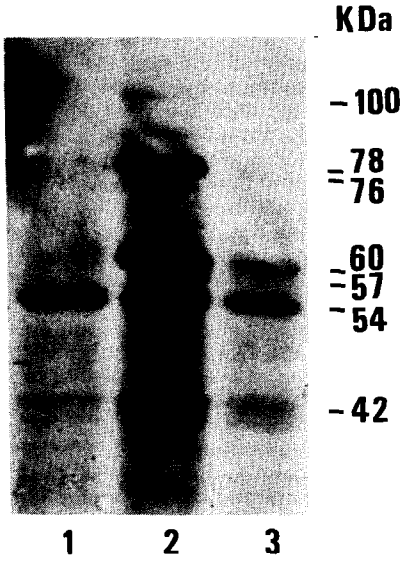

Fig. 4. Detection of TFA-antigens in halothane-exposed human hepatocytes by immunoblotting. Cultured human hepatocytes were exposed to halothane for 4 hr (lane 3), 24 hr (lane 2) or incubated without anaesthetic for 24 hr (lane 1). Whole cell homogenates were prepared and 100 μg protein per lane was separated on a 10% polyacrylamide gel. Proteins were transferred electrophoretically to a nitrocellulose membrane and immunoblots were developed using anti-TFA–RSA serum: The molecular masses of the major polypeptides recognized by the anti-TFA–RSA serum are indicated.

TFA-neoantigens generated as a result of oxidative biotransformation of the anaesthetic. Sera of HH patients as well as hapten-specific anti-TFA antibodies reacted with TFA-adducts in liver tissue or in isolated hepatocytes from *in vivo* halothane-exposed rabbits, guinea pigs and rats [2, 15, 19]. Moreover, both patients' sera and anti-TFA-antibodies recognized several polypeptides of similar molecular weights in liver microsomes of halothane-treated animals in immunoblotting analysis [6].

These results clearly indicate that isolated human hepatocytes exposed to halothane in culture can generate halothane-related neoantigens as shown by immunolocalization and immunoblotting. Several observations support the conclusion that formation of neoantigens recognized with anti-TFA antibodies in cultured hepatocytes results from halothane metabolism. First, previous data from this laboratory have demonstrated that human hepatocytes retained *in vitro* high levels of cytochromes P450, inducible phase I and phase II drug metabolizing enzymes and heterogeneous cellular distribution of cytochrome P450 enzymes for several days [20, 21]. Second, the generation of TFA-antigens was shown in livers of halothane-treated animals, predominantly in centrilobular areas [15, 19]. Third, the metabolism of halogenated anesthetics such as halothane and enflurane is catalysed mainly by cytochrome P450 2E1 [22, 23], which is preferentially expressed in centrilobular hepatocytes in both non-induced and induced livers [24, 25]. Moreover, the zonal functional characteristics of both periportal and centrilobular hepatocyte subpopulations and, in particular, their different capacities to metabolize xenobiotics have been reported to be preserved for several days in primary culture [26, 27]. The differential expression of TFA-antigens in halothane-exposed human hepatocytes might reflect the heterogeneous distribution of P450 2E1 and consequently the different halothane metabolic capacities of these cells according to their intralobular distribution *in vivo*. Neoantigens were mainly localized in the endoplasmic reticulum in which P450 enzymes were located. Similarly, halothane-induced antigens in rabbit livers after exposure *in vivo* to halothane were concentrated in the microsomal fraction [28]. In addition, TFA-adducts were expressed on the surface of human hepatocytes, supporting previous studies with rat hepatocytes [15]. Thus, intact hepatocytes might induce an immune response in susceptible patients exposed to halothane.

Further characterization of halothane-induced neoantigens with anti-TFA antibody revealed the specific expression of neoantigens with apparent molecular masses of 100, 78, 76, 60/59, 57 and 42 kDa in human hepatocytes exposed *in vitro* to halothane. Immunoblotting studies performed by Kenna *et al.* [6] demonstrated that hapten-specific anti-TFA antibody recognized different liver microsomal proteins which were expressed only in the livers of halothane-treated rats. The most intensely stained fractions (100, 76, 59, 57 and 54 kDa) corresponded closely to the protein fractions recognized by circulating antibodies from HH patients. Incubation of guinea pig liver slides under the atmosphere of 95% O_2/5% CO_2 containing 1.7 mM halothane for 12 hr resulted in the appearance of five distinct neoantigens with molecular weights of 97, 62, 57, 54 and 51 kDa [29]. Finally TFA-liver protein antigens of 100, 76 and 57 kDa were detected in liver samples of two patients exposed to halothane [13]. The results presented here indicate that neoantigens generated in cultured human hepatocytes after *in vitro* exposure to halothane are very similar to neoantigens identified in livers of *in vivo* halothane-treated animals. Several of these TFA-proteins have been purified and characterized from liver microsomal fractions of halothane-treated rats. A 57 kDa antigen was shown to be a TFA-modified form of protein disulphide isomerase [30] whereas antigens of 59 and 63 kDa have been identified as TFA-labelled forms of liver microsomal carboxylesterase isoenzyme [31] and of the calreticulin, respectively [32]. A 58 kDa antigen appeared to be highly homologous to modified phosphatidylinositol-specific phospholipase C-α [33] and a 100 kDa antigen corresponded to a TFA-labelled form of ERp99 or endoplasmin [34]. The 60/59 kDa protein fraction of human hepatocytes was the most extensely stained with anti-TFA–RSA serum and may be related to the 63/59/58 kDa TFA-antigens found in rat liver. The 78, 76 and 42 kDa antigens also appeared to be abundant in halothane-treated hepatocytes whereas the 100 kDa polypeptide was induced at a relatively weaker extent. In addition, it was found that anti-TFA antibody cross-reacted with a polypeptide of 54 kDa in control hepatocytes. This recognition was abolished with pre-immune

rabbit sera. The cross-reaction of specific anti-TFA antibody with polypeptides in hepatocyte homogenates obtained from rats not treated with halothane was reported by Christen *et al.* [35]. In conclusion, the findings of this study demonstrate that: (1) primary human hepatocytes can metabolize halothane *in vitro* and generate TFA-neoantigens; (2) exposure to halothane resulted in formation of TFA-antigens in all tested human hepatocyte cultures indicating that antigen expression may be a common event in humans exposed to halothane and is not restricted to patients with HH; (3) TFA-adducts were expressed mainly in the endoplasmic reticulum and also on the plasma membrane of halothane-treated hepatocytes that make them accessible to immune effector mechanisms in intact hepatocytes *in vivo*; (4) neoantigen formation varied markedly among different donors. It was believed that the level of TFA-antigen expression may be one of the important factors contributing to its immunogenicity [36]. Thus, primary culture of human hepatocytes represents a unique experimental model for studying the generation of halothane-induced neoantigens and influence of factors effecting their expression under well-defined conditions and for determining the relevance of *in vitro* and *in vivo* investigations on animal hepatocytes to the human situation.

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