The molecular basis of halothane-induced hepatitis

J. Gerald Kenna
Department of Pharmacology and Toxicology, St Mary's Hospital Medical School, Norfolk Place, London W2 1PG, U.K.

Halothane hepatitis
The anaesthetic agent halothane (CF₃CHClBr) causes two distinct types of liver damage. About 20% of halothane-exposed patients exhibit mild hepatotoxicity, characterized by transient elevations in liver enzymes. In contrast, a much smaller fraction of patients exposed to halothane (between 1 in 3500 and 1 in 35,000) develop severe hepatotoxicity, commonly termed 'halothane hepatitis', which frequently may progress to liver failure and death [11]. Typically, patients with halothane hepatitis tend to be obese females in late middle age, although a minority of patients are not obese, some are male and the syndrome has been described in children [2]. In common with many other adverse drug reactions which are of clinical importance, halothane hepatitis is idiosyncratic and dose-independent. The underlying pathogenic mechanisms have yet to be resolved unequivocally, although various clinical and laboratory findings indicate that immune processes are involved [1]. A series of recent investigations have outlined a likely mechanism, which involves an immune response to a group of novel, halothane metabolite-modified liver protein antigens.

Immune response to halothane-induced liver antigens
Initially, Vergani et al. [3] found that leucocytes from patients with halothane hepatitis were sensitized to undefined components present in homogenates of livers from halothane-anaesthetized rabbits, but not in homogenates from ether-anaesthetized rabbits, as assessed using a crude in vitro leucocyte migration test. Subsequently, indirect immunofluorescence and induced cellular cytotoxicity studies demonstrated that the patients' sera contained antibodies to antigens present on hepatocytes isolated from livers of halothane-anaesthetized rabbits, but not on hepatocytes from untreated or ether-anaesthetized rabbits [4]. These studies showed that the halothane-induced antigens were expressed on the surface membrane of isolated rabbit hepatocytes and that rabbit hepatocytes bearing the novel antigens were susceptible to antibody-directed cytotoxic killing by normal human lymphocytes in vitro. In addition, antibodies to the antigens were detected only in sera from patients with halothane hepatitis and not in a wide range of control sera, including sera from patients who received multiple halothane anaesthetics but did not develop liver damage, and sera from patients who sustained hepatitis attributable to recent viral infection and who had undergone recent anaesthesia with halothane. More recent studies, performed using other methods for antibody detection (e.g., I.s.a. and immunoblotting) [5, 6], have confirmed that antibodies to halothane-induced liver antigens are restricted to patients with halothane hepatitis. Thus sensitization to halothane-induced antigens is not simply a secondary consequence of halothane exposure, or of halothane exposure plus liver damage.
The halothane-induced antigens are trifluoroacetylated liver proteins

The nature of the halothane-induced antigens was investigated by SDS-PAGE and immunoblotting. A group of five polypeptide antigens (100, 76, 59, 57 and 54 kDa) were identified which were recognized by antibodies from sera of patients with halothane hepatitis and were expressed in livers of halothane-treated rabbits and rats, but not in livers of control animals [6, 7] (Fig. 1). Patients' sera differed markedly in patterns of polypeptide antigen recognition (Fig. 1), with the 100 and 76 kDa antigens being recognized most commonly.

Subcellular fractionation studies revealed that, in livers of halothane-treated rabbits, each of the halothane-induced polypeptide antigens was concentrated in the microsomal fraction [6]. Halothane is metabolized in the liver by the microsomal cytochrome P-450 enzyme system [8]. Two distinct pathways have been described, which yield reactive species that bind covalently to liver microsomal proteins and lipids. Oxidative metabolism to trifluoroacetyl halide (TFA-halide) is favoured at high oxygen tensions and at normal oxygen tensions, while reductive metabolism to the 1-chloro-2,2,2-trifluoroethyl radical is favoured at low oxygen tensions [8-10]. A detailed investigation of the relationship between halothane metabolism and antigen expression has been undertaken [7]. This showed that the antigens recognized by the patients' antibodies correspond in apparent molecular mass to the major TFA-labelled proteins present in livers of halothane-treated rats. Detection of TFA-labelled proteins was achieved by immunoblotting, using anti-TFA antiserum from rabbits immunized with TFA-rabbit serum albumin [11, 12] (see Fig. 1).

Also, antigen expression was markedly decreased when rats were treated with deuterated halothane (CF₂CDClH₇) rather than halothane. Deuteration of halothane decreases the rate of oxidative metabolism but does not decrease the rate of reductive metabolism [9]. In addition, the antigens were produced in vitro when rat liver microsomal fractions were incubated with halothane and NADPH under aerobic conditions, but not when the incubations were performed anaerobically [7]. These findings demonstrate that antigen generation involves oxidative metabolism of halothane to TFA-halide.

Recognition of the antigens by the patients' antibodies was abolished when liver microsomal fractions from halothane-treated rats were incubated with 1 M-piperidine, to remove covalently bound TFA groups. However, binding of the patients' antibodies to the antigens was inhibited only partially by addition of a high concentration (50 mM) of the hapten inhibitor TFA-L-lysine [7]. It appears that the novel epitopes recognized by the patients' antibodies contain covalently bound TFA groups but do not comprise simply the TFA group. Most probably, the epitopes consist of the TFA group together with structural features which are unique to the various protein carriers, so that each polypeptide antigen is recognized as an immunochemically distinct entity.

Antigen expression in human liver

To investigate whether halothane-induced antigens are produced in humans anaesthetized with halothane, livers from patients who had died of cardiac failure shortly after cardiac surgery were analysed by immunoblotting [13]. Blots were developed using sera from four patients with halothane hepatitis, which contained antibodies to the 100, 76 and 57 kDa TFA-antigens. The sera reacted strongly with antigens of very similar apparent molecular
masses which were expressed in livers from two patients who had received halothane, but not in normal human liver or in livers from three patients who had received other anaesthetics. The human antigens were not recognized by antibodies present in various control sera. Recognition of the 100 kDa and 76 kDa human liver antigens by the patients' antibodies was greatly reduced by incubation of sera with liver microsomal fractions from halothane-anaesthetized rabbits, but not by incubation with control rabbit liver microsomal fractions. These results indicate that humans exposed to halothane express TFA-liver protein antigens which are analogous to the antigens produced in halothane-treated rabbits and rats and are recognized by antibodies present in sera of humans who have developed halothane hepatitis. Presumably, it is the human antigens which, in susceptible patients, represent the immunogens which elicit immune responsiveness.

Since halothane hepatitis is extremely rare, it is highly unlikely that the two patients in whose livers the antigens were detected would have developed halothane hepatitis had they not died of post-operative cardiac failure. Thus it is probable that antigen expression occurs in all humans exposed to halothane and not just in patients who develop halothane hepatitis. Certainly, TFA-antigens are produced reproducibly in all halothane-exposed rabbits [6] and rats [7]. In contrast, in only very few humans do the TFA-protein antigens act as immunogens.

Antigen expression in rats

To gain insight into factors which may influence expression of the TFA-antigens in halothane-anaesthetized humans, the influence of various factors upon antigen expression in rats was investigated, using the immunoblotting method [14]. An implicit assumption is that the properties of the antigens recognized by human antibodies are similar in rats and humans.

In rats treated intraperitoneally with halothane, the 100, 76, 59 and 57 kDa antigens were scarcely detectable at 3 h after halothane treatment, but were expressed in maximal amounts at 12 h and remained detectable after 7 days [14]. The long lifetimes of this group of antigens may help to explain how they elicit immune responses in susceptible patients, particularly since the 100, 76, 59 and 57 kDa antigens were the major long-lived TFA-protein conjugates detected in livers of halothane-treated rats [14]. However, other considerations must be involved also, since patients differ in patterns of antigen recognition (Fig. 1). The 54 kDa antigen was expressed in maximal amounts at 3 h after halothane treatment and was scarcely detectable by 12 h [14]. Also, the 54 kDa antigen was expressed in much greater amounts than the 100, 76, 59 and 57 kDa antigens when rat liver microsomal fractions were incubated with halothane in vitro [7]. It appears that the 54 kDa TFA-antigen is produced more rapidly than the other antigens but is much more short-lived. Its relatively short lifetime may explain why antibodies to the 54 kDa antigen are present in patients' sera much less frequently than are antibodies to the 100, 76, 59 and 57 kDa antigens [6, 7].

The various antigens were not detectable in kidney, lung, fat, brain or skeletal muscle but were expressed in testis, although at far lower levels than in liver [14]. Thus antigen expression is relatively liver-specific. This is consistent with the liver antigens being the immunogens which elicit the immune response and also with the hypothesis that the antigens are the targets of immune-mediated liver injury in patients in vivo.

When rats were treated with cytochrome P-450-inducing agents prior to administration of halothane, expression of the 54 kDa antigen was increased by treatment with β-naphthoflavone, decreased by phenobarbitone and isosafrole and relatively unaffected by isoniazid or clofibrate [14]. However, expression of the 100, 76, 59 and 57 kDa antigens was decreased by treatment with β-naphthoflavone, phenobarbitone and isosafrole, increased by isoniazid and either unaffected (100 kDa) or moderately decreased (76, 59 and 57 kDa) by clofibrate. The contrasting results obtained with phenobarbitone and isoniazid are of interest since both agents induce oxidative metabolism of halothane [8, 15]. The decreased expression of the 100, 76, 59 and 57 kDa antigens seen in phenobarbitone-treated animals was accompanied by a marked increase in TFA-labelling of microsomal proteins of about 54 kDa, which were not recognized by patients' antibodies and which most probably correspond to the major cytochrome P-450 isoenzyme(s) induced by phenobarbitone [12]. Presumably, these proteins trap TFA-halide very efficiently, perhaps due to their very high abundance in microsomal fractions of phenobarbitone-treated rats (6-7% of total microsomal protein [16]) and/or high intrinsic reactivity, so that relatively little TFA-halide is available for reaction with other proteins. Perhaps this does not occur with isoniazid because isoniazid causes a much more modest induction of cytochrome(s) P-450 [17]. Similar con-
siderations may explain the results obtained with the other agents. Regardless of the explanation, these results suggest that expression of the 54 kDa antigen may involve metabolism of halothane by cytochrome P-450 isoenzyme(s) which are distinct from the form(s) involved in generation of the other antigens. In addition, they raise the possibility that TFA-antigen expression may vary markedly in halothane-anaesthetized humans, as a consequence of inter-individual variability in hepatic cytochrome P-450 isoenzyme profile.

**Purification and biochemical characterization of antigens**

The TFA-59 kDa antigen was purified from liver microsomal fractions of halothane-treated rats by immunoaffinity chromatography on a column of anti-TFA IgG and anion-exchange h.p.l.c. Based upon its N-terminal amino acid sequence, catalytic activity and biochemical properties, the antigen was identified as a TFA-labelled liver microsomal carboxylesterase isozyme (EC 3.1.1.1) [18]. Subsequently, a strategy was developed which made possible purification of the other major TFA-antigens, in relatively high yields. This involved selective solubilization of the antigens from liver microsomal fractions by extraction with 0.1% sodium deoxycholate buffer, followed by column chromatography on DEAE-Sepharose and hydroxylapatite h.p.l.c. [19]. A combination of amino acid sequence analysis and biochemical properties has indicated that the 57 kDa antigen corresponds to TFA-labelled protein disulphide isomerase (EC 5.4.3.1) [19] and that the 100 kDa antigen is a TFA-labelled form of a stress protein, termed variously ERp99, GRP94 or endoplasmic [20]. During the antigen purification work, two additional TFA-antigens (63 kDa and 58 kDa) were identified which were not resolved from TFA-59 kDa by one-dimensional SDS/PAGE. Deduced amino acid sequence information, obtained from cDNA that had been cloned following detection by an antiserum to the purified 63 kDa antigen, has revealed that the TFA-63 kDa antigen is a TFA-modified form of a calcium-binding protein described very recently, termed calreticulin [21].

Liver microsomal carboxylesterase, protein disulphide isomerase, ERp99 and calreticulin share several properties. Each protein is thought to be a soluble protein present within the lumen of the endoplasmic reticulum. Each protein is very abundant (roughly 1% or more of total microsomal protein). None of the proteins metabolize halothane. A possible mechanism by which the antigens may be generated is illustrated schematically in Fig. 2. Biochemical studies have confirmed that the 100, 57 and 59/58/63 kDa TFA-antigens, and also the TFA-76 kDa antigen, are situated within the lumen of microsomal vesicles prepared from livers of halothane-treated rats (J. G. Kenna, unpublished work). Diffusion of TFA-halide across the lipid bilayer is speculative, but seems plausible. Interaction of TFA-halide with the proteins may be quite nonspecific and a consequence of their high relative abundance, or the proteins may have high intrinsic reactivities with TFA-halide. Specific interaction with particular cytochrome P-450 isoenzyme(s) and/or with each other is possible. An interesting feature of the model is that it predicts that the TFA-

---

**Fig. 2**

Possible mechanism of generation of TFA-labelled polypeptide antigens

Halothane is metabolized by cytochrome(s) P-450 to TFA-halide on the cytoplasmic face of the endoplasmic reticulum. This highly reactive species reacts indiscriminately with proteins. The major target is cytochrome P-450, although TFA-halide also diffuses across the lipid bilayer and labels a group of proteins situated on the luminal side of the membrane. Note that at least two of the antigens (59 and 57 kDa) are believed to be dimers; this is not shown.
protein antigens might be translocated from the endoplasmic reticulum to the hepatocyte surface membrane by the normal processes of membrane flow.

**Mechanism of halothane hepatitis**

A working hypothesis which is consistent with the results reviewed above is shown in Fig. 3. This scheme emphasizes the limitations of our current knowledge. For example, very little information is available regarding likely mechanisms of immune-mediated hepatocyte damage, other than the original antibody-dependent cellular cytolysis results [4]. In particular, we have little idea of why only a very small fraction of patients mount an immune response to the TFA-antigens and develop hepatitis, whereas all halothane-exposed patients (and animals) appear to produce the antigens. As discussed previously, qualitative and quantitative variability in halothane metabolism and antigen generation may be important, as may variability in expression of the antigens on the hepatocyte surface membrane. Only antigens which are expressed on the cell surface should be accessible to immune effector mechanisms in intact hepatocytes *in vivo*, and expression on the cell surface may be required for initial immune sensitization. Variability in the immune response might arise as a consequence of variability in factors such as antigen presentation, immune recognition and immunoregulation. Now that individual TFA-antigens are being purified and specific antibodies to the antigens are being produced, it should be possible to design experiments which address many of these important issues.

**Fig. 3**

Postulated mechanism of immune-mediated halothane hepatotoxicity

Hepatocyte + halothane

\[ \downarrow \]

1. Metabolism and covalent binding

Trifluoroacetylated microsomal proteins

\[ \downarrow \]

2. Translocation (membrane flow?)

Expression on hepatocyte surface

\[ \downarrow \]

3. Immune sensitization

Immune response

\[ \downarrow \]

4. Immune destruction

Hepatotoxicity

This work was supported by an Advanced Training Fellowship in Toxicology from the Wellcome Trust.


Received 10 September 1990