

# Oxidative metabolism of halothane in the production of altered hepatocyte membrane antigens in acute halothane-induced hepatic necrosis

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**SUMMARY** Previous investigations have shown that patients with fulminant hepatic failure after halothane anaesthesia have a circulating antibody which reacts with an antigen present on the surface of halothane-altered hepatocytes. In the present study, it has been shown that the expression of the antigen is associated with the oxidative metabolism of halothane, in contrast with results of other groups which have shown that the reductive route is involved in the direct hepatotoxic reaction attributed to halothane.

Although a direct hepatotoxic effect of halothane has been shown in experimental animals,<sup>1-3</sup> many clinical and laboratory features of the severe hepatic necrosis which follows halothane anaesthesia suggest an immunological component.<sup>4</sup> Patients with this condition can be shown to be sensitised to hepatocyte membrane components the antigenicity of which has become altered by halothane or a metabolite,<sup>5</sup> and, in other recent studies using immunofluorescence techniques, we have demonstrated that serum from these patients contains antibodies which react specifically with halothane-altered hepatocytes.<sup>6</sup> This antibody is not found in patients with normal liver function tests after multiple exposure to halothane, nor in any other form of liver damage. *In vitro*, these antibodies were also shown to be capable of rendering these hepatocytes susceptible to lysis by normal lymphocytes. The appearance of such drug-altered liver antigens may be linked with the production of chemically reactive metabolites. Halothane is metabolised *via* at least two different pathways, oxidative and reductive, depending on the ambient oxygen tension. Both pathways generate reactive metabolites which bind to cellular macromolecules.<sup>7</sup> Experimental models, pointing to a direct toxic action of the drug, have shown that the lesion is associated with the reductive pathway of halothane metabolism.<sup>12</sup> In the present study, we have investigated the importance of both these pathways of halothane metabolism in the expression of the altered hepatocyte antigen.

## Methods

### PREPARATION OF HALOTHANE-ALTERED HEPATOCYTES

To circumvent the difficulty of preparing the appropriate antigen *in vitro*, we used hepatocytes isolated from rabbits previously exposed to halothane, in which metabolism of the drug and antigenic alterations could take place *in vivo*.

Female New Zealand white rabbits, weight 2.5 kg, were used throughout. Anaesthesia was induced with 3% halothane in 97% oxygen and subsequently maintained for 45 minutes with 1% halothane in various fixed oxygen/nitrogen mixtures (see below). Animals were allowed to recover and 18 hours later were killed, the livers removed, and hepatocytes isolated for the cytotoxicity assay (see below).

Stimulation of the oxidative pathway of halothane metabolism was achieved by maintaining anaesthesia in 99% oxygen, while the reductive pathway was preferentially stimulated by administration of the agent in 14% oxygen/85% nitrogen. Two other groups of animals were anaesthetised with intermediate oxygen tensions (40% oxygen/59% nitrogen and 25% oxygen/74% nitrogen).

Preliminary studies showed that pretreatment with phenobarbitone alters the spectral binding characteristics of halothane to isolated rabbit liver microsomes in a way which would favour reductive metabolism of the drug, while administration of  $\beta$ -naphthoflavone (BNF) stimulates the oxidative pathway. Two other groups of rabbits were pretreated with either pheno-

barbitone 30 mg/kg intraperitoneally for three days or with a single dose of BNF 80 mg/kg intraperitoneally 48 hours before anaesthesia with 1% halothane in 99% oxygen.

#### CYTOTOXICITY ASSAY

The halothane-altered hepatocyte antigen was detected by the indirect cytotoxicity assay in which sera from patients known to contain the antibody which reacts with the halothane-altered liver membrane determinant were incubated with hepatocytes isolated from rabbits anaesthetised with halothane under different conditions. In the presence of the altered antigen the antibody will bind to the cell and render it susceptible to cytotoxicity by normal lymphocytes in the cytotoxicity assay.

Eighteen hours after anaesthesia the rabbit was killed; the liver was removed and then perfused with RPMI 1640 medium. The liver was then minced and isolated hepatocytes prepared by digestion with collagenase (Boehringer-Mannheim). The cells were maintained in short-term culture in tissue culture flasks suspended in RPMI 1640 medium with 10% fetal calf serum (Difco Laboratories). Twenty-four hours before the assay the hepatocytes were seeded into microtest culture plates (Falcon 3034) to give approximately 100 cells per well, and maintained at 37°C in an atmosphere of 95% oxygen and 5% carbon dioxide. The tissue culture fluid was aspirated and 10 µl RPMI containing either 1% test serum absorbed with normal hepatocytes or 10% fetal calf serum was added to at least 10 wells. The plates were then incubated for two hours at 37°C. The supernatant was aspirated and 10 µl culture medium added. This was again aspirated and replaced with 10 µl lymphocytes obtained from healthy individuals by dextran sedimentation, cotton-wool incubation (to remove macrophages), and Ficoll-Triosil centrifugation, and suspended in RPMI 1640 medium with 10% fetal calf serum. The ratio of lymphocytes to hepatocytes was 100:1. After 36 hours' incubation at 37°C in an atmosphere of 95% oxygen and 5% carbon dioxide, the plates were inverted for two hours and then washed with medium. The number of remaining hepatocytes was counted and the percentage cytotoxicity determined with the formula:

$$\% \text{ cytotoxicity} = \frac{(\text{Mean no. hepatocytes in control wells}) - (\text{Mean no. hepatocytes in test wells})}{\text{Mean no. hepatocytes in control wells}} \times 100$$

where the controls consist of hepatocytes incubated with 10% fetal calf serum and then lymphocytes.

#### Results

None of the sera induced significant cytotoxicity

Table Cytotoxicity induced by sera from patients with halothane-related hepatic necrosis

| Pretreatment     | Oxygen concentration (%) | No. of sera cytotoxic |
|------------------|--------------------------|-----------------------|
| Nil              | 99                       | 7/7                   |
| Nil              | 14                       | 0/7                   |
| β-Naphthoflavone | 99                       | 7/7                   |
| Phenobarbitone   | 99                       | 0/7                   |

against hepatocytes from the control group of animals, whereas this was observed with all seven sera when hepatocytes isolated from rabbits anaesthetised with halothane in 99% oxygen were used ( $p < 0.01$ , Student's paired *t* test) (Table). This effect was no longer seen with hepatocytes from animals anaesthetised under hypoxic conditions with 14% oxygen and 85% nitrogen. The three sera giving the highest cytotoxicity values were used in additional tests with hepatocytes from animals anaesthetised at intermediate oxygen tensions. Significant cytotoxicity was induced against hepatocytes isolated from rabbits anaesthetised at oxygen concentrations of 40% and above, but not at oxygen tensions of 25% and below (Figure). There was a progressive fall in cytotoxicity values with decreasing oxygen tension.

After pretreatment with the enzyme-inducing agents phenobarbitone or β-naphthoflavone and subsequent anaesthesia with halothane in 99% oxygen, significant cytotoxicity was observed in all seven sera when β-naphthoflavone was used but this effect was not seen after phenobarbitone (Table).

#### Discussion

The present studies have clearly shown that the appearance of the halothane-altered hepatocyte surface membrane antigen is associated with the oxidative pathway of metabolism. The findings that the circulating antibodies which react with the antigen were present in the patients with severe hepatic necrosis after halothane anaesthesia suggest that a similar, if not identical, altered membrane determinant was generated during anaesthesia and that this was produced by the oxidative route. How exposure to halothane leads to alteration of hepatocyte membrane antigens is not yet established. Anaesthetic agents have been shown to alter membrane fluidity<sup>8</sup> and, as a result, antigenic determinants, which are not normally accessible to circulating components of the immune system, could be exposed. Alternatively, a chemically reactive metabolite, binding to the smooth endoplasmic reticulum could, according to the membrane flow hypothesis,<sup>9</sup>

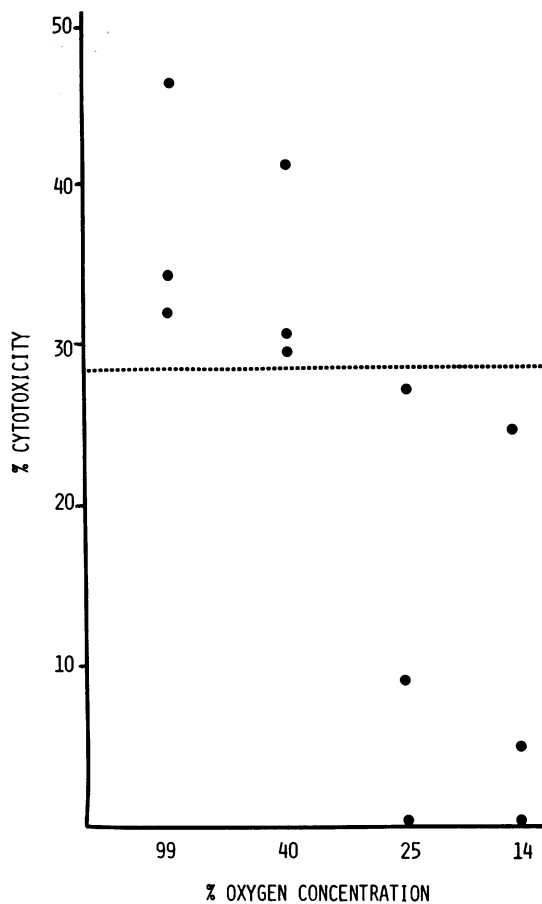


Figure Effect of oxygen tension on induced cytotoxicity to halothane-treated hepatocytes. Dotted line represents upper limit of normal.  $r = -0.81$  (Spearman's rank correlation test)  $p < 0.01$ .

become translocated to the cell surface and become incorporated as a hapten into the plasma membrane.

The involvement of the oxidative pathway in the generation of altered membrane determinants contrasts with what has been demonstrated with respect to halothane metabolism and the mechanism of the direct hepatotoxic effect. Here, studies of Sipes,<sup>1</sup> Cousins,<sup>2</sup> and Reynolds<sup>3</sup> in experimental animals have shown that reactive metabolites generated by the reductive route are implicated in the hepatic damage. Minor abnormalities in liver function tests, associated with mild focal parenchymal necrosis, have been reported in up to 20% of patients repeatedly exposed to halothane.<sup>10,11</sup> The relative frequency of this condition, coupled with the absence of any evidence of sensitisation to halothane-altered hepa-

toxicity components, which we have recently documented,<sup>12</sup> suggests that it may arise as a result of direct toxicity. Quantitative differences in the metabolism of the drug *via* the reductive route, influenced by genetic or environmental factors, could form the basis of susceptibility to this lesion, but this requires documentation. In contrast with the mild lesion, severe hepatic necrosis is associated in many patients with sensitisation to halothane-altered liver cell components,<sup>6</sup> suggesting that, as well as any direct toxic action of the drug, a superadded immunological component is required. The relative contributions of direct hepatotoxicity and immunological mechanisms could vary from patient to patient. This could explain the spectrum of hepatic lesions observed,<sup>13</sup> with variable proportions of centrilobular necrosis, suggestive of direct toxicity, and peripheral lymphocyte infiltration, suggestive of an immune reaction.

Patient susceptibility to the immunological component of the disease is unlikely to be due to individual differences in the metabolism of the drug *via* derivatives which alter hepatocyte antigens, because the oxidative route is the major pathway of halothane metabolism in man.<sup>14</sup> Furthermore, evidence of the altered antigen has been obtained in all rabbits exposed to halothane in oxygen tensions greater than 40%. If this also applies in man, then the occurrence of demonstrable sensitisation only in those few who develop a severe hepatic necrosis suggests that the basis of the lesion may be an abnormal immune system. In keeping with this is the high frequency of antithyroid and anti-liver/kidney microsomal autoantibodies in such patients.<sup>4</sup>

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