

Hepatotoxicity of General Anesthetics on Rat Hepatoma Cells in Culture¹ (39308)

YOSHIAKI GOTO,² CARLOS A. DUJOVNE,³ DON W. SHOEMAN,
AND KASUMI ARAKAWA
(Introduced by C. D. Klaassen)

Clinical Pharmacology and Toxicology Center, Departments of Anesthesiology, Medicine and Pharmacology, University of Kansas Medical Center, College of Health Sciences and Hospital, Kansas City, Kansas 66103

There is a great deal of controversy on the mechanism and incidence of hepatotoxicity of general anesthetics. The toxicity of these drugs in experimental animals is not necessarily relevant to that in man and the evaluation of the hepatotoxicity in humans is hampered by the low incidence and the difficulty of evaluation in postoperative patients.

Previous demonstration of a positive correlation between the cytotoxicity *in vitro* and the clinical hepatotoxic potential *in vivo* for other therapeutic drugs (1-3) prompted an investigation of the cytotoxic effect on cell cultures derived from rat hepatoma of three marketed anesthetic agents: ethyl ether,⁴ halothane (H),⁵ methoxyflurane (MF),⁶ and of an investigational one, isoflurane (IF).⁷

Methods. Liver cell cultures (rat hepatoma CCI 144, The American Type Culture Collection) were purchased from Gibco Laboratories (Rhode Island, N. Y.). Cells were grown in monolayers by standard techniques in HAMS F-12 medium (Gibco Lab-

oratories)⁸ supplemented with glutamine and 10% fetal bovine serum on the glass surfaces of 16 × 125 mm screwcap tubes incubated in a horizontal position at 37°. Monolayers were grown until reaching total confluency at which time each culture tube contained approximately 10⁶ cells. Cell counts were performed by standard techniques in a Coulter Counter B apparatus. Four to six culture tubes taken randomly from the batch of cultures prepared for the cytotoxicity experiments on the same day were counted. Cell counts in every tissue culture tube after the cytotoxic experiment were precluded by differences in cytolysis after toxicity from the various drugs; consequently, replicate cultures (rather than very same cultures used for cytotoxicity) were used for cell counts.

Cell counts were found to be 10⁶ ± 10⁵ cells per tube. Drug concentrations in tissue cultures were arbitrarily expressed in micrograms per 10⁶ cells since there were no significant differences in tissue's drug concentrations among tubes of the same stock containing cells within this range of variability and exposed to the same drug and concentration.

To begin the toxicity experiment the culture medium of each tube was discarded and the monolayers which are attached to the glass were rinsed with 1 ml of balanced salt solution. Eight milliliters of a solution containing 1 or 1.5 μl/ml of the respective anesthetic in medium Parker 199 (TCM) was added; TCM without drugs was used in the controls. The pH of the different solutions was between 7 and 7.4; differences in this magnitude were not found to produce

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² Dr. Goto was a research fellow in anesthesiology and clinical pharmacology. His current address is Tokyo Medical College, Tokyo, Japan.

³ Dr. Dujovne was a recipient of a Pharmaceutical Manufacturers Association Foundation Faculty Development Award, 1972-1974.

⁴ Mallinckrodt, St. Louis, Mo.

⁵ 2-Bromo-2-chloro 1,1,1-trifluoroethane, Fluothane.

⁶ 2,2-Dichloro-1,1-difluoroethyl methyl ether, Penthrane.

⁷ 1-Chloro-2,2,2-trifluoro-ethyl difluoromethyl ether, Forane, Ohio Medical Products, Murray Hill, N. J.

⁸ The composition of this and any other media mentioned are detailed in Grand Island Biological Co. catalog and manual, 1974-1975.

changes in the cells or in the results of these types of experiments. All procedures up to this point were performed with aseptic techniques. After incubation at 37° for 5 hr the culture tubes were centrifuged for 30 min at 600g. The supernatant was removed, kept in an airtight vial filled to capacity to prevent air dissipation of the drug, and saved frozen for later drug assay. An aliquot of the remaining supernatant was used for enzyme assay immediately. One milliliter of 0.25% trypsin was introduced into the culture tubes. They were incubated at 37° for 30 min and frozen and thawed five times with brisk agitation in between to completely lyse the cells. The cell lysate was kept frozen and sealed until assayed for drug content.

Cytotoxicity was quantitated by assay of enzymes leaked from cells into surrounding culture media after exposure to the drugs. The activities of lactate dehydrogenase (LDH) and β -glucuronidase (BG) in TCM, were expressed in conventional units per milliliter of culture media and determined as described in previous reports (2, 3). Activation and/or leakage of lysosomal enzymes from hepatocytes have been recognized as a useful parameter of cell injury produced by hepatotoxins and viruses *in vivo* and *in vitro* (4, 5).

In view of the volatility of the drugs, precautions were taken to keep tissue culture tubes and all other containers tightly capped and to maintain a uniform and minimum time during which they were open for manipulation. All liquid and air compartments were kept at the same volume and contact with adsorptive materials such as rubber or plastic was avoided. Three to five replicate cultures were used for each drug in each experiment and every experiment included simultaneous exposure to three to four drugs.

The concentrations of anesthetic agents in media and cell lysates obtained as described above were assayed by gas chromatography on a 5 ft \times 1/4-in. aluminum column packed with 80/100 mesh chromosorb 102 operated at the following temperatures: column 155°, detector 275°, and inlet 180°. Four microliters of media or cell lysate were injected directly.

Statistical analysis of the results was done

by regression analysis of dose response curves, analysis of variance, and Student's *t* tests.

The following experiments were performed to make certain that changes in enzyme activities were not due to chemical interference of the drugs on the enzymes or the assays per se: Medium used in controls or medium containing drugs in solutions at the concentrations used in the experiments were used to dilute animal sera containing LDH and BG activity. Aliquots of media containing enzymes' activities after the cytotoxicity experiments were also exposed to each one of the drugs at the concentrations used. The activities of LDH and BG were again determined at 0 and 5 hr of incubation. No differences in activities among drugs or controls were found.

Gross cytopathic effects of the drugs on the cell cultures were evaluated by appearance under light phase microscopy. Also, vital staining was performed on some parallel experiments to verify the capacity of some of the drugs to affect the viability of the cells. After exposure to drugs or controls to 5 hr, the media was discarded as usual and 2 ml of Trypan blue 0.15% was introduced for 10 min. Dead cells acquire a blue color but living cells have the capacity to exclude the dye.

Results. Morphological changes. The appearances of gross changes in cell morphology and viability were used only to corroborate that enzyme leakage was secondary to cytotoxic effects from the drugs. Cytopathic changes alone were not as useful as the activities of leaked enzymes in media to determine dose-dependent differences in toxicity among all drugs. Nevertheless, at the end of 5 hr it was possible to differentiate between cultures which were exposed to E or IF and those exposed to H or MF. The latter drugs produced intense cytoplasmic granulation, blurring of cell borders, shrinking of some cells, and complete necrosis of others (Fig. 1). Vital staining of cultures showed a greater number of dead cells in those exposed to H or MF than of those exposed to E or IF or controls. There were no differences in number of dead cells (which were very scanty) in cultures of these latter two groups.

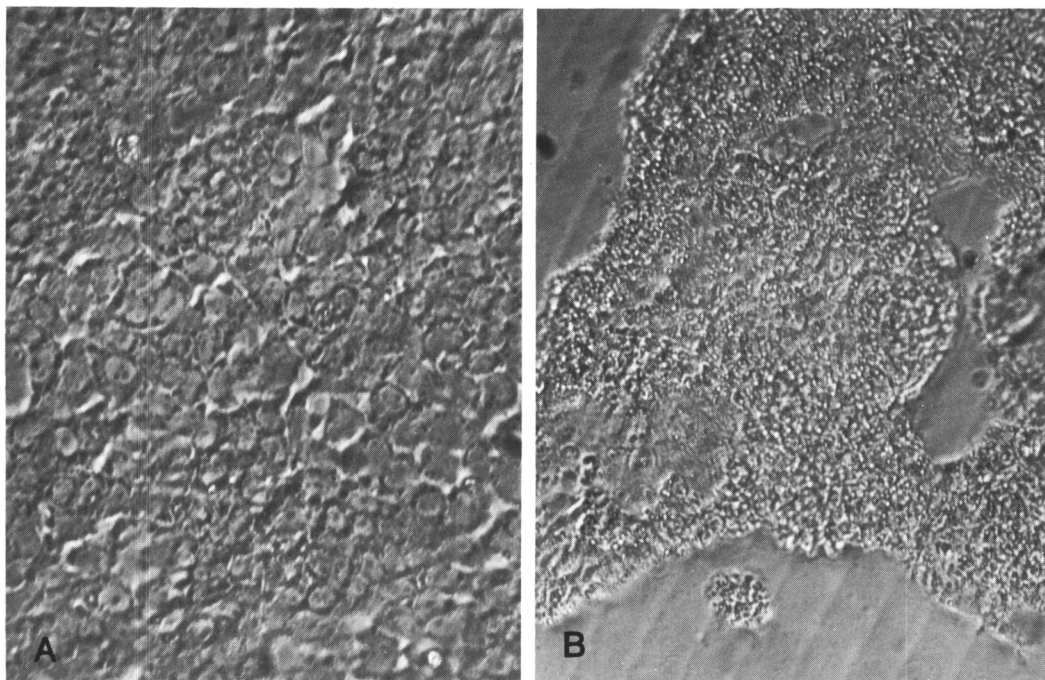


FIG. 1. Cultures of rat hepatoma cells (CCL 144) after 5 hr of incubation with control media (A) or media containing 1.5 $\mu\text{l/ml}$ halothane (B). Phase contrast, $\times 240$.

Enzyme leakage. Since all these drug preparations were liquids the only possible and consistent dosing *in vitro* was by weight or volume. We have chosen to refer the cytotoxic effect to the original dose in microliters of drug per milliliter of media (Figs. 2 and 3).

Linear regression curves of loss of LDH (a soluble cytoplasmic enzyme) and BG (a lysosome enzyme) after exposure of cell cultures to the anesthetics at doses of 0.5, 1.0, and 1.5 $\mu\text{l/ml}$ of media for 5 hr are shown in Figs. 2 and 3. The greatest leakage of LDH and BG was found after exposure to MF; progressively decreasing enzyme loss was seen in media of cultures exposed to H and IF. Ether exerted a cytotoxic effect no greater than controls. The differences among all drugs were statistically significant ($P < 0.01$).

Drug concentrations. These drugs are volatile and differences in the degree of cytotoxicity could have been due to different concentrations as a result of differences in media/air or media/cells partition among them. The concentration of the drugs in the cellular and surrounding environment was

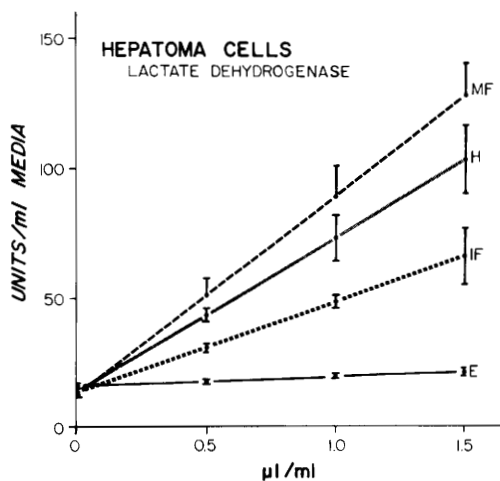


FIG. 2. Linear regression curves of lactate dehydrogenase activity in culture media of hepatoma cells after exposure to 0.5, 1, and 1.5 $\mu\text{l/ml}$ of the anesthetics for 5 hr. Each drug different from each other ($P < 0.01$); 35 to 50 cultures were exposed to each drug.

measured at the end of many of the experiments (Table I). The drugs are listed in order of their increasing cytotoxic effect so it can be easily appreciated that it does not necessarily correspond with the order of in-

creasing concentrations attained in cells or media. MF, which had the greatest cytotoxic effect, was the least concentrated in cells.

From another set of experiments we calculated the enzyme leakage in relation to drug concentration in cells (Fig. 4). Expressing toxicity as units of enzymes leaked per milliliter of media per microgram of drug inside the cells, at lower drug concentrations only MF exerted significant toxicity and at higher ones MF, H, and IF were all more cytotoxic than E. Nevertheless, the overall comparative effects among drugs remained approximately the same, further supporting the fact that the differences in toxicity were due to intrinsic cytotoxic potential of the drugs and not to possible differences in the

capacity of drugs to reach the cell membranes.

Discussion. MF, H, and IF, in decreasing order of magnitude, produced toxic effects on cultures of cells derived from rat liver parenchymae. MF and H produced the greatest cytotoxicity, IF caused toxicity which was greater than or similar to that due to E, depending on the concentrations used, and E exerted negligible toxicity. The concentration of the anesthetics in the media ranged from 157 to 598 $\mu\text{g/ml}$ which correspond to the reported surgical anesthetic concentrations of these drugs in blood taken from the hepatic vein of experimental animals during anesthesia (6).

It must be stressed that *in vitro* studies with volatile drugs require uniform and controlled techniques regarding exposure to air spaces or adsorptive surfaces. The determination of actual concentrations of the drugs in the system at the termination of some of the experiments was essential to draw valid conclusions. The final concentrations of these drugs in the same volumes of the liquid media or cells used could vary depending on the physicochemical properties of the particular drug (i.e., partition coefficient).

Ether anesthesia has not been reported to be hepatotoxic in man (7). Isoflurane is a new investigational anesthetic drug, chemically related to ether, alleged to produce less abnormality on liver function than H or MF in human volunteers (8). The available clinical and pathological data strongly suggest that H and MF can produce hepatic necrosis in man (9).

We should point out that the comparative *in vitro* effects observed with the four drugs

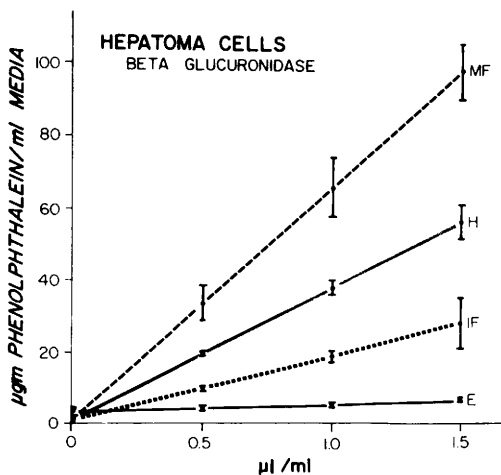


FIG. 3. Linear regression curves of beta glucuronidase activity in culture media of hepatoma cells after exposure to 0.5, 1, and 1.5 $\mu\text{l/ml}$ of the anesthetics for 5 hr. Each drug different from each other ($P < 0.01$); 35 to 50 cultures were exposed to each drug.

TABLE I^a

Initial dose ($\mu\text{l/ml}$ of media)	Concentrations at 5 hr							
	In media ($\mu\text{g/ml}$)				In cell ($\mu\text{g}/10^6$)			
	E	IF	H	MF	E	IF	H	MF
0.5	220 ± 30	182 ± 20	196 ± 20	157 ± 20	76 ± 10	75 ± 11	82 ± 15	50 ± 6
1.0	421 ± 30	281 ± 20	468 ± 30	470 ± 30	97 ± 9	80 ± 15	129 ± 13	86 ± 11
1.5	590 ± 30	492 ± 30	598 ± 40	552 ± 50	140 ± 7	83 ± 11	153 ± 17	107 ± 14

^a Concentration of drugs measured in aliquots of media and cell lysates after exposure to 0.5, 1.0, and 1.5 $\mu\text{l/ml}$ of TCM for 5 hr. Means \pm SE of 10 to 40 cultures exposed to each drug at each concentration.

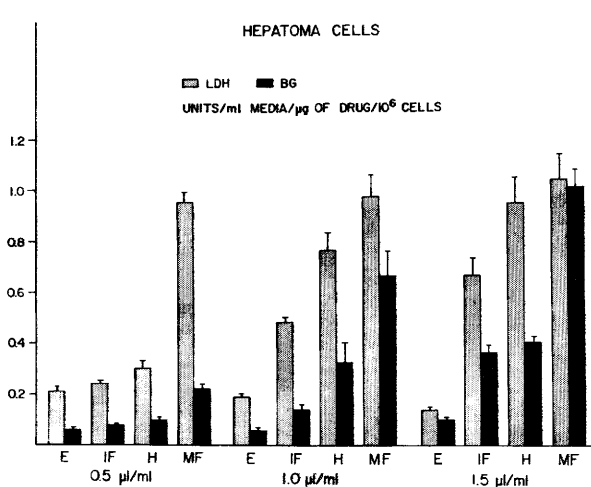


FIG. 4. Mean and SE of activities of lactate dehydrogenase (LDH) and beta-glucuronidase (BG) per milliliter of media per microgram of drug in 10^6 cells (6 to 24 cultures exposed to each drug at each concentration). The following were statistically significant ($P < 0.01$): 0.5 $\mu\text{l/ml}$, MF different from E, IF, or H; 1.0 $\mu\text{l/ml}$, each drug different from each other; 1.5 $\mu\text{l/ml}$, each drug different from each other except IF from H (BG) and H from MF (LDH).

studied paralleled what is currently known about their hepatotoxic potential in man. *In vitro* tissue culture experiments may not reflect reactions or responses in the whole organism; consequently, the connection between our results and the *in vivo* experience should be interpreted with due reservation and a true correlation remains to be proven.

The role of metabolites of H (trifluoroethanol or trifluoroacetate) and MF (methoxydifluoroacetate or dichloroacetate) in producing hepatotoxicity in man or animals is controversial (10). The cells used in these experiments derived from an established culture line from the American Type Culture Collection. They were originally grown from a transplantable Morris hepatoma; they can synthesize albumin and exert other functions which are rather exclusive of hepatocytes such as production of serum complement and conjugation and/or metabolism of bilirubin, testosterone, and *para*-aminophenol (11-13). It is not known if these cells have the capacity to metabolize any of the drugs used in this study; if they do, the possibility exists that it may differ from that of human hepatocytes. Nevertheless, these cultures could be useful for evaluation of acute "direct" drug toxicity on cellular membranes with the advantage of con-

trolled environment and drug concentrations at the cellular level.

The mechanism of MF or H hepatotoxicity is not known and the incidence is very low, probably less than 1 in 10,000 exposures. Allergic or hypersensitivity mechanisms have been postulated by some and rejected by others (14-16). Our results support the view that these halogenated anesthetics exert a direct toxic effect on cellular membranes of organs where, as in the liver, they are sufficiently concentrated. It is of interest that MF, which is an hepatotoxic as well as a nephrotoxic drug in man (17), was the most toxic to the cell cultures *in vitro*. The greater cytotoxicity of H and MF was not a consequence of a greater concentration of these drugs in the tissues, suggesting that the difference in toxicity derived from the individual cytotoxic potency of the drugs.

In vitro, the cytotoxic anesthetics had a predictable and widespread effect while the effects in man are known to be unpredictable and of variable extent. We think this difference could arise from the fact that the concentrations of the drugs *in vitro* are controlled and kept constant. The concentrations of these anesthetics in the liver or bile of patients have not been measured to our

knowledge; however, for many other drugs it is known that administration of similar doses of the same drug can result in a wide range of intrahepatic and biliary concentrations among different individuals.

Injury to organs *in vivo* depends on the tissue levels attained, but also may be modified by "individual idiosyncrasy" of the host. It is conceivable that just a few individuals can attain a given concentration of the drugs in the cellular environment necessary to have an hepatotoxic effect or that only "hypersensitive" individuals can develop toxicity at such concentrations.

This laboratory has reported data demonstrating a correlation between *in vitro* tissue culture cytotoxicity and the *in vivo* incidence of hepatotoxicity for other therapeutic drugs such as phenothiazines (1), erythromycin (2), and oxiphenisatin (3). Moreover, similar comparative cytotoxic effects of these anesthetics was demonstrated in Chang liver cells derived from human liver and will be the subject of a forthcoming report from this laboratory. Nevertheless, the reliability of data derived from *in vitro* studies of this type to predict the hepatotoxic potential of anesthetic drugs remains to be established.

Summary. Rat liver hepatoma cells in monolayer tissue cultures were exposed to various concentrations of ether (E), isoflurane (IF), halothane (H), or methoxyflurane (MF) for 5 hr. Loss of intracellular enzymes into surrounding media was used to quantitate cytotoxic effects. Drug concentrations in cells and media were monitored during some of the experiments. Dose- and concentration-related cytotoxic effects were evident for all drugs. The order of cytotoxic potency was MF > H > IF > E. The differences were not related to the intracellular concentrations accomplished by each drug. The direct dose-related toxic effects of MF and H on the cell membranes suggest that other than hypersensitivity factors may play a role in the hepatotoxicity from these halogenated anesthetics. The correlation between the *in vitro* effects on liver cells and

the suggested comparative hepatotoxic potentials of these drugs in man supports the possible usefulness of this method to gather additional information on comparative hepatotoxic potential of general anesthetics.

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